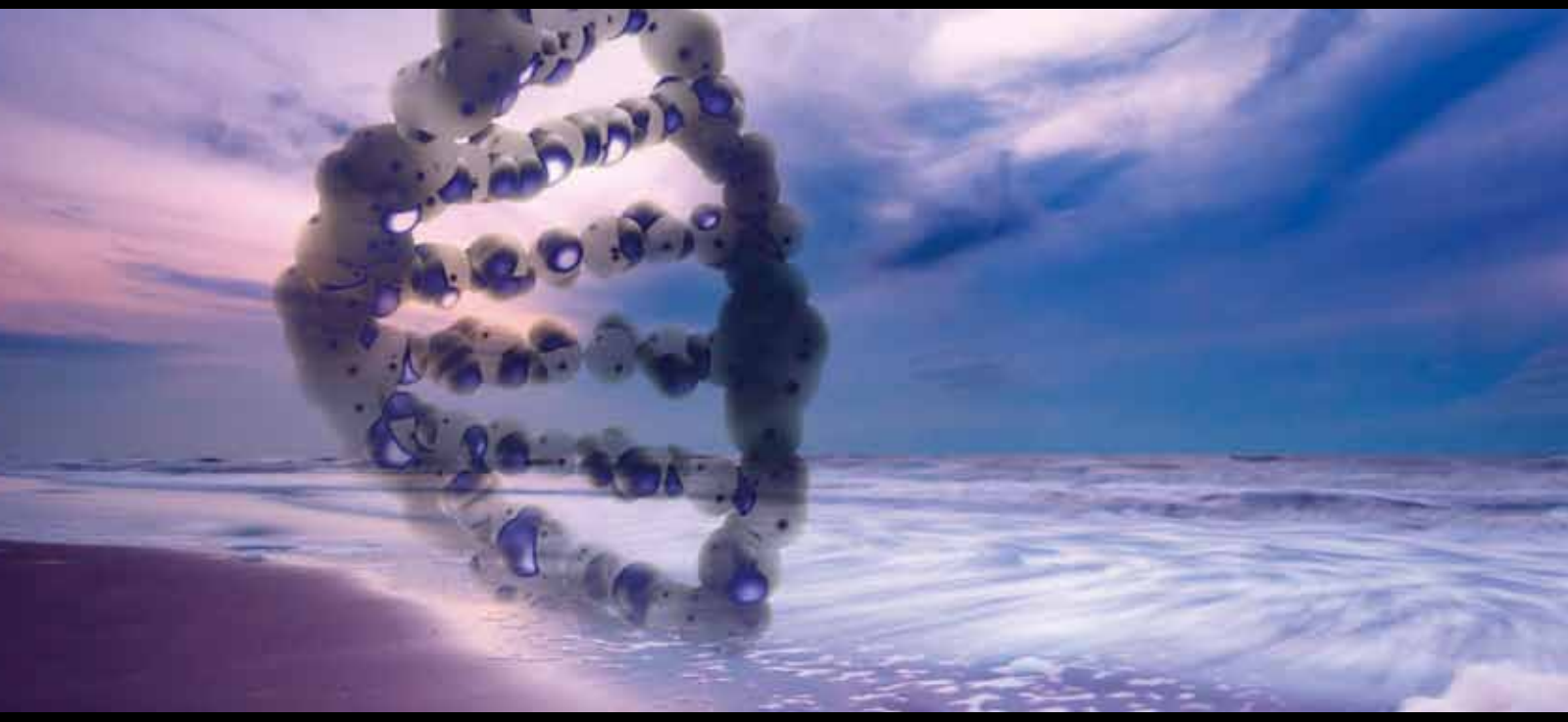
International Journal of Evolutionary Biology

# Mechanisms of Speciation

Guest Editors: Kyoichi Sawamura, Chau-Ti Ting, Artyom Kopp, and Leonie C. Moyle



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# Editorial Board

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# *Editorial*
# Mechanisms of Speciation

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"Speciation" was largely "speculation" two decades ago, at least with respect to a detailed and comprehensive mechanistic understanding of the origin of new species. Despite elegant classical work examining the genetic basis of interspecific differences and reproductive isolation and complementary studies of the ecological factors that can contribute to species divergence, speciation researchers lacked the tools to dissect the specific forces, traits, and genes involved. Thanks to the recent advances in molecular biology and genomic sequencing, detailed study of speciation is becoming feasible in many animal and plant groups. In fact, a dozen of "speciation genes" responsible for reproductive isolation between sibling species have been identified at the molecular level. Further, genetic changes leading to morphological differentiation among related species have been elucidated, supported by phylogenetic analyses at high resolution. We invited investigators to contribute both original research and review articles that would stimulate the continuing efforts to understand speciation and species differentiation from all perspectives, not only the genetic mechanisms but also the ecological and evolutionary causes.

Among the seven original articles in this special issue, four were *Drosophila* studies. Two of them focus on the mechanisms of reproductive isolation. A. Takahashi et al. in "*Cuticular hydrocarbon content that affects male mate preference of Drosophila melanogaster from west Africa*" identified a polymorphic chemical cue involved in mate recognition between sibling species. Y. H. Ahmed-Braimah and B. F. McAllister in "*Rapid evolution of assortative fertilization between recently allopatric species of Drosophila*" described an example of postmating/prezygotic isolation, where heterospecific fertilization after mating is compromised due to disruptions in sperm storage and motility. Often, reproductive isolation evolves more rapidly than any morphological traits so that the only way to distinguish recently diverged species is through mating experiments. Y.-F. Li et al. in "*DNA barcoding and molecular phylogeny of Drosophila lini and its sibling species*" showed that molecular variation can also be widely shared between sibling species despite strong reproductive isolation between them. To understand why reproductive isolation can evolve so rapidly compared to other traits, L. Müller et al. in "*Inter- and intraspecific variation in Drosophila genes with sex-biased expression*" examined the evolution of gene expression and protein sequences and found that genes with male-biased expression tend to diverge rapidly compared to the rest of the genome.

Three other original articles deal with reproductive isolation in vertebrates, specifically fish or reptiles. D. Bierbach et al. in "*Divergent evolution of male aggressive behavior: another reproductive isolation barrier in extremophile poeciliid fishes*?" reported a rare case study of behavioral isolation via local adaptation to extreme environmental conditions (darkness in caves and toxic hydrogen sulphide). G. M. Kozak et al. in "*Postzygotic isolation evolves before prezygotic isolation between fresh and saltwater populations of the rainwater killifish, Lucania parva*" discovered a case of incipient reproductive isolation caused by salinity adaption, in which they found no evidence of prezygotic isolation but detected reduced hybrid survival. M. Gabirot et al. in "*Differences in chemical sexual signals may promote reproductive isolation and cryptic speciation between Iberian wall lizard populations*"

reported another case of cryptic speciation caused by pheromonal differentiation, in which they demonstrated that the Iberian wall lizard forms part of a "species complex" with different morphology and different proportions of chemical components in femoral gland secretions of males.

This special issue also includes four review articles. A. Ivanović et al. in "*A phenotypic point of view of the adaptive radiation of crested newts* (*Triturus cristatus superspecies, Caudata, Amphibia*)" reviewed the pattern of adaptive radiation in the European crested newt, in which they suggested that phenotypic diversification was caused by heterochronic changes linked to variation in ecological preferences. J. P. Masly in "*170 years of "lock-and-key": genital morphology and reproductive isolation*" reviewed the facts and speculations about the role of genital morphology in maintaining species barriers and examined the prospects for identifying the genetic changes responsible for the evolution of genital morphology. D. M. Castillo and L. C. Moyle in "*Evolutionary implications of mechanistic models of TE-mediated hybrid incompatibility*" reviewed mechanistic models of host-mediated TE suppression in light of the potential role of TE derepression in postzygotic isolation and identified data that would be necessary to provide more satisfactory tests of this hypothesized isolation mechanism. K. Sawamura in "*Chromatin evolution and molecular drive in speciation*" proposed a general mechanism of hybrid sterility and inviability caused by coevolution between repetitive satellite DNAs and chromatin proteins.

Altogether, this is a diverse array of papers that ranges from a classical model of speciation research—*Drosophila*—to plants and emerging vertebrate system, and from molecular genetic mechanisms through sensory biology to environmentally mediated adaptive divergence. As such, this special issue is representative of the diversity of studies and systems that continue to contribute to our understanding of speciation and the diversity of mechanisms that surely underlie this fundamental evolutionary process. We can only hope that the field, and our understanding, continues to grow so diversely and creatively in the future.

## Acknowledgments

We would like to express our gratitude to all the authors and reviewers who contributed to the success of this special issue.

*Kyoichi Sawamura*
*Chau-Ti Ting*
*Artyom Kopp*
*Leonie C. Moyle*

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*Research Article*

# Cuticular Hydrocarbon Content that Affects Male Mate Preference of *Drosophila melanogaster* from West Africa

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Intraspecific variation in mating signals and preferences can be a potential source of incipient speciation. Variable crossability between *Drosophila melanogaster* and *D. simulans* among different strains suggested the abundance of such variations. A particular focus on one combination of *D. melanogaster* strains, TW1(G23) and Mel6(G59), that showed different crossabilities to *D. simulans*, revealed that the mating between females from the former and males from the latter occurs at low frequency. The cuticular hydrocarbon transfer experiment indicated that cuticular hydrocarbons of TW1 females have an inhibitory effect on courtship by Mel6 males. A candidate component, a C25 diene, was inferred from the gas chromatography analyses. The intensity of male refusal of TW1 females was variable among different strains of *D. melanogaster*, which suggested the presence of variation in sensitivity to different chemicals on the cuticle. Such variation could be a potential factor for the establishment of premating isolation under some conditions.

## 1. Introduction

*Drosophila* exhibits complex mating behavior with frequent wing vibration and copulation attempts by males. The successful mating is achieved by communications between males and females using chemical, acoustic, and visual signals (reviewed in [1]). Subtle differences in these signals may accumulate during or after the formation of reproductive isolation. Once reproduction isolation is established to a certain extent, the correct mate recognition is essential to avoid costly hybridization and wasting time on unsuccessful courtship. Indeed, a certain degree of premating isolation or mating incompatibility is commonly observed between closely related species of *Drosophila* [2, 3].

In some cosmopolitan species of *Drosophila*, for example, *D. ananassae* [4] and *D. elegans* [5, 6], widely observed mating incompatibilities between populations from different locations exist. The degree of incompatibility is variable among sampled strains in these species. Another cosmopolitan species, *D. melanogaster*, also harbors incompatible combinations of populations [7–11]. The degree of incompatibility between populations is also variable, and many intermediate strains are typically observed. These within species incompatibilities suggest that there are many intraspecific variations in mating signals and preferences. Those variations could either fix in isolated populations or become targets of sexual selection under some conditions and consequently result in divergent mating-associated characters

among different populations. It is important to understand the precise features of such variations in signals and perceptions that could potentially lead to an incipient speciation.

Cuticular hydrocarbons are known to play an important role as a contact pheromone in *Drosophila* mate recognition (reviewed in [12, 13]). They are used to recognize conspecific mate [14–16], as well as to distinguish sex [17–19] and to evaluate the mating history of the females by males [20–22]. In *D. melanogaster*, many studies have shown that some female specific long chain hydrocarbons, especially C27 dienes, are attractive substances for males, and some of the male components, *cis*-vaccenyl acetate (cVA) and 7-tricosene (7:C23), are shown to reduce the female attractiveness [12, 13]. Furthermore, "pheromone-free females" were courted by males more rigorously than wild-type females [16, 23], which highlights the importance of both attractive and inhibitory components for the courtship induction [24].

The quantity of each cuticular hyrdrocarbon component varies among closely related species of *D. melanogaster* [25] and even within this species [26]. Also, the profile could be modified by different food conditions as shown in cactophilic *D. mojavensis* [27]. Therefore, making correct decisions based on variable contents of cuticular hydrocarbons may not be a trivial task. Thus, the differential sensitivity to different hydrocarbon components may evolve relatively fast and could be a source of variations for differential mate choice.

In this study, we analyze within-species variations in mate preference using *D. melanogaster*, whose mating-associated characters and their genetic bases are extensively studied. We particularly focus on one combination of conspecific strains that show low mating frequency, and investigate the role of cuticular hydrocarbon on this intraspecific mating incompatibility. Also, by comparing gas chromatograms of different strains, we attempt to identify candidate components in female cuticular hydrocarbon that have an inhibitory effect on mating.

## 2. Materials and Methods

*2.1. Drosophila Strains.* Five *D. melanogaster* inbred strains, 4 *D. simulans* inbred strains, and 3 *D. simulans* isofemale lines were used in this study. Mel6(G59), TW1(G23), KY02001(G20), BZ1(G23), and VAV1(G15) are wild-derived *D. melanogaster* inbred strains originated from Benin (West Africa), Taiwan, Japan, Brazil, and Tonga, respectively [30–32]. W86(G15), sim CH1(G65), sim 5(G69), and SEY4(G51) are wild-derived *D. simulans* inbred strains originated from Madagascar, USA., Congo, and Seycheleis Islands, respectively. Numbers in the parentheses indicate numbers of generations sibmated in the laboratory. S2 is a *D. simulans* isofemale line from Japan, which has been reported to be highly crossable to *D. melanogaster* [33]. Mel7 and Mel8 are *D. simulans* isofemale lines originated from Benin (West Africa). Flies were fed with regular sugar-yeast food and cultured at 23–25°C under a natural laboratory light condition.

*2.2. No-Choice Design for Interspecific Mating Experiment.* Interspecific no-choice mating experiment was conducted by placing females and males of different species together in a test vial. Unmated females and males were collected after lightly anesthetized with $CO_2$ and were used 2 days after eclosion. Ten females from one species and 10 males from another species were put into a test vial (2.7 cm diameter × 10 cm height) filled with ~2.5 cm food at the bottom and capped with a sponge plug. The females were dissected after 24 hours to score the presence or absence of sperm in the spermatheca and/or seminal duct. Up to 2 occasional losses of samples were permitted per test vial. All the mating tests were done in the morning hours in a room temperature (~23°C) under a natural laboratory light condition.

*2.3. Double-Choice Design.* Double-choice mating experiment was conducted by placing females and males from 2 different strains together in a test vial. Unmated females and males were collected after lightly anesthetized with $CO_2$ and were used 4 days after eclosion. Ten females and 5 males each from both strains were placed in a test vial (2.7 cm diameter × 10 cm height) filled with ~2.5 cm food at the bottom and capped with a sponge plug. The copulated pairs were aspirated out of the vial during the 3-hour test period and kept until the combinations of the mated strains were scored. Nine replicate test vials were scored for each experiment. The identification of strains was done using thoracic trident pigmentation intensity or abdominal pigmentation pattern. All the mating tests were done in the morning hours in a room temperature (~23°C) under a natural laboratory light condition.

*2.4. No-Choice Design for Intraspecific Mating Experiment.* Intraspecific no-choice mating experiment was conducted by placing one type of females and another type of males into a test vial. Unmated females and males were collected after lightly anesthetized with $CO_2$ and were used 4 days after eclosion. Ten females and 10 males were placed in a test vial (2.7 cm diameter × 10 cm height) filled with ~2.5 cm food at the bottom and capped with a sponge plug. The number of copulated pairs was counted by aspirating them out of the vial during the 1-hour test period. Six replicate test vials were scored for each experiment. All the mating tests were done in the morning hours in a room temperature (~23°C) under a natural laboratory light condition.

*2.5. Cuticular Hydrocarbon Transfer Experiment.* Cuticular hydrocarbon transfer experiment was done by rub-off method as in Coyne et al. [14]. Cuticular hydrocarbon was transferred from TW1 to Mel6 by crowding 100 TW1 virgin females and 10 Mel6 virgin females in a limited space (2.7 cm diameter × 1 cm height) of a food vial for 4 days. Then the flies were lightly anesthetized with $CO_2$ to select out Mel6 females. The thoracic trident pigmentation intensity was used to identify Mel6 females. The efficient transfer of cuticular hydrocarbon by this method was confirmed by gas chromatography (GC, data not shown). The control sets of females were obtained by crowding 110 Mel6 virgin females in the same limited space for the same period of time. Those

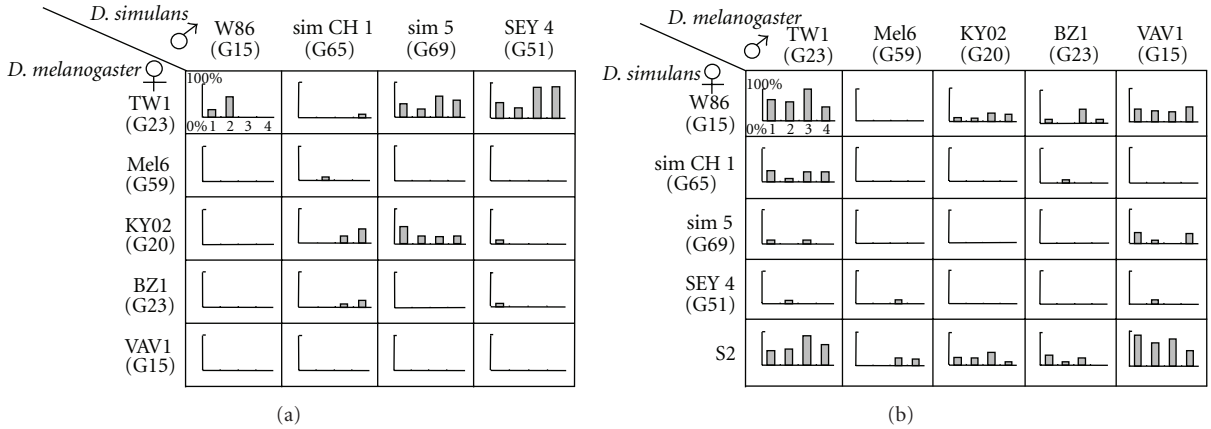


Figure 1: Mating frequency between strains from *D. melanogaster* and *D. simulans* by no-choice design. Vertical axis of each graph indicates % inseminated females after 24 hours. Each bar indicates result from a test vial. The first 2 replicate vials and the last 2 replicate vials were tested on a separate day. (a) Mating frequency between *D. melanogaster* females and *D. simulans* males. (b) Mating frequency between *D. simulans* females and *D. melanogaster* males.

females were used within 30 minutes after separated from the crowd.

*2.6. Cuticular Hydrocarbon Analyses.* Cuticular hydrocarbons were extracted from 4-day-old virgin females by n-hexane and analyzed by gas chromatography (GC). Cuticular hydrocarbons were extracted by washing 5 individuals in a glass culture tube with 500 $\mu$L *n*-hexane for 5 min at room temperature. Then, *n*-hexane solution was transferred to a glass spits tube and stored in $-20^{\circ}$C after the solvent was fully evaporated. Immediately before GC analyses, 100 $\mu$L *n*-hexane was added to the tube, and 2 $\mu$L of the resultant solution (equivalent to extract from ~0.1 individual) was used for the analyses. GC analyses were performed with a Shimazu GC-14A equipped with a DB-1 apolar column (length, 30 m; diameter, 0.25 mm; film thickness, 0.25 $\mu$m; Agilent Technology Inc.) and flame ionization detector. Helium was used as the carrier gas. Injection was made in splitless mode for 1 minute at 300°C with a detector temperature of 300°C. The oven was programmed to hold at 80°C for 1 minute, and increased at 10°C/minute to 320°C, and held for 5 minutes.

## 3. Results

Premating isolation among *D. melanogaster* sibling species is not complete. For example, a certain level of mating occurs between *D. melanogaster* and *D. simulans* in the laboratory. If there are slight intraspecific differences in visual, acoustic, or chemical signals during the mating and differences in perception and response to those signals, they would show up as differential mating frequencies to a closely related species. In order to unveil these differences, we surveyed mating frequencies between different strains of *D. melanogaster* and *D. simulans*.

The experiments were done by interspecific no-choice design, which involves placing 10 females and 10 males from different species into a test vial. The mating frequencies were assessed by scoring the number of inseminated females after 24 hours. For each cross, 2 vials were tested each day for 2 days, which gave 4 replicate data in total. Mating frequencies within 24 hours between the 2 species were not very high, but varied among strains (Figure 1).

Among those interspecific crosses, most notable differences were found between TW1 and Mel6. TW1 females readily mated with males from most of the *D. simulans* strains tested (Figure 1(a)), whereas females from Mel6 as well as those from VAV1 rarely mated with males from *D. simulans* strains. TW1 males also mated frequently with females from many of the *D. simulans* strains tested (Figure 1(b)), but Mel6 males seldom mated with *D. simulans* females. The mating patterns in most of other strains fell in between those two strains (Figures 1(a) and 1(b)). By the same experimental setting with 10 females and 10 males in each of the two test vials, 18 (90%) out of 20 Mel6 females mated with Mel6 males and 16 (84%) out of 19 TW1 females mated with TW1 males within 2 hours. The results indicated that individuals from Mel6 strain mated readily with individuals from their own strain but seemed to avoid mating with *D. simulans*.

Taken together, these results indicated that TW1 and Mel6 indeed have differential mating signals or responses to those signals or both. We also noticed during the experiments that Mel6 males rarely exhibited courtship behavior to *D. simulans* females when placed together in a same vial. This observation suggested that the male mate preference could be different between TW1 and Mel6.

Since frequencies of successful mating with *D. simulans* differed between TW1 and Mel6, we suspected that the difference in mate preference may affect the mating between the two strains. In order to investigate whether the mating occurs randomly between the two strains, we first conducted mate choice experiment by double-choice design, which involves placing females and males from both strains

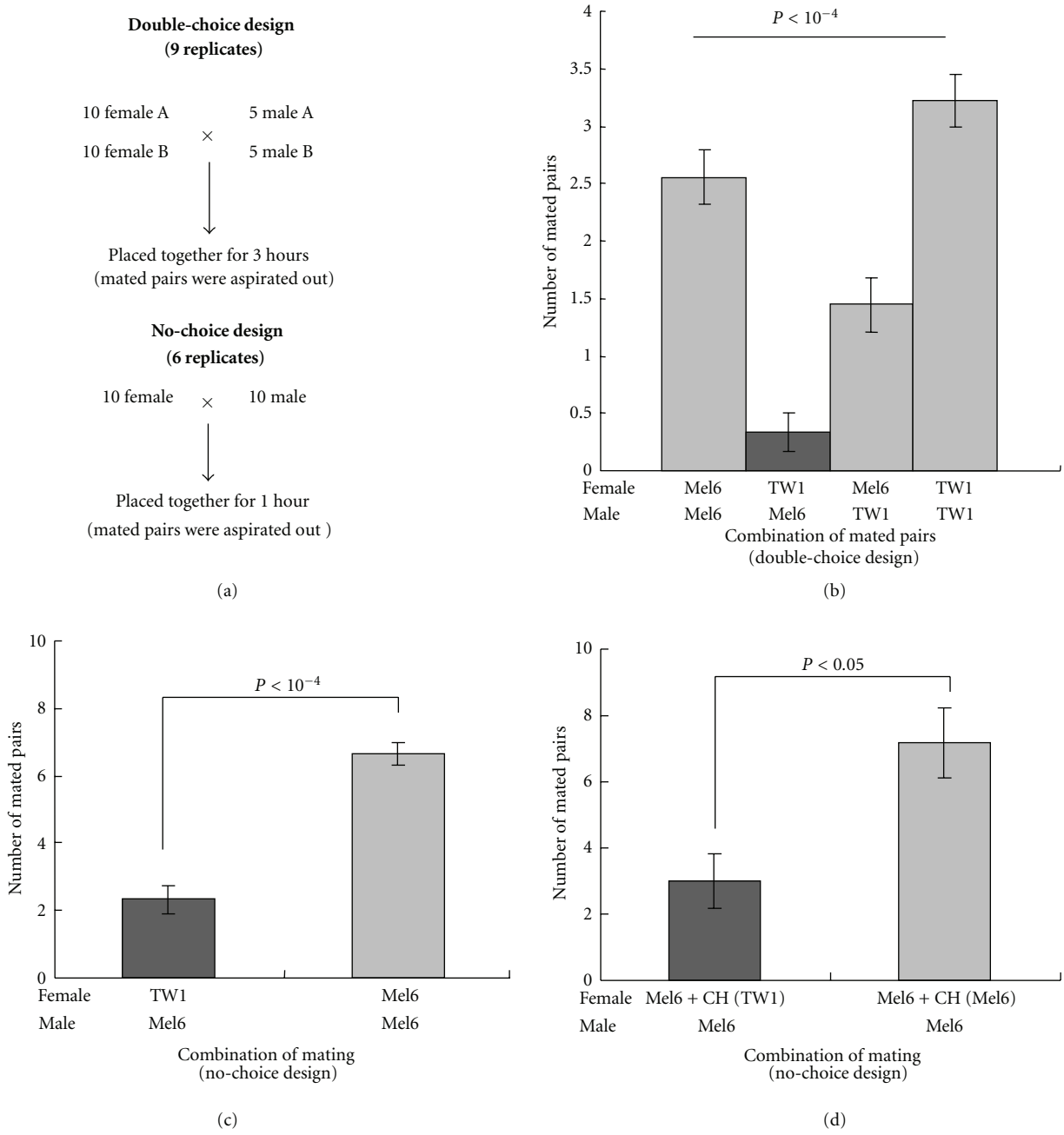


Figure 2: Mating experiment between TW1 and Mel6 by double-choice and no-choice design. (a) Description of the mating designs. (b) Number of mated pairs in the double-choice mating experiment. The pattern deviated from random mating (Cochran-Mantel-Haenszel exact test $P < 10^{-4}$). (c) Number of mated pairs in the no-choice mating experiment between TW1 female and Mel6 male and that between Mel6 females and males. The difference between the two combinations of strains was significant (Student $t$-test, $t = 7.27$, df = 10, $P < 10^{-4}$). (d) Number of mated pairs in the no-choice mating experiment between Mel6 males and Mel6 females coated by cuticular hydrocarbon of TW1 females, "Mel6 + CH(TW1)", and that between Mel6 males and Mel6 females coated by cuticular hydrocarbon of their own, "Mel6 + CH (Mel6)". The difference between the two combinations was significant (Student $t$-test, $t = 3.08$, df = 10, $P = 0.012$).

together in a test vial (Figure 2(a)). As we suspected, the mating pattern deviated from random choice (Figure 2(b), Cochran-Mantel-Haenszel exact test ($P < 10^{-4}$), and indicated that the mating between TW1 female and Mel6 male occurred at a very low frequency.

Together with the observation that Mel6 males rarely courted *D. simulans* females, we decided to focus our attention to mate preference by Mel6 male. First, the no-choice design was adopted, where only one type of females and one type of males were placed together in a mating vial

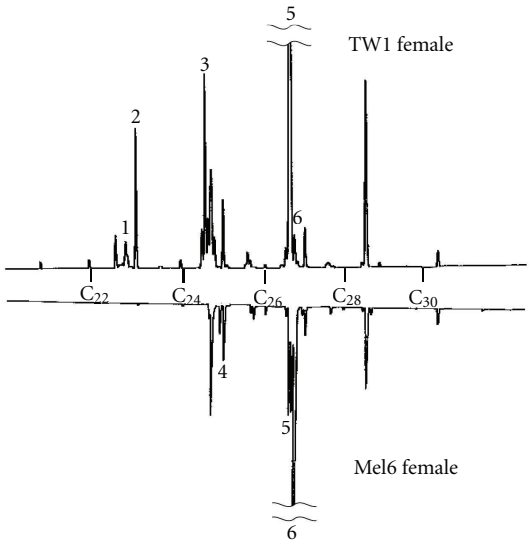


Figure 3: Mirrored gas chromatograms of *n*-hexane extracts of TW1 and Mel6 females. $C_{22}$–$C_{30}$ standard marker positions are indicated between the chromatograms. Each peak corresponds to hydrocarbon compound whose identity could be inferred from the previous studies [28, 29]. The peaks indicating qualitative differences (presence/absence or extreme high/low) between the two strains are marked by numbers. The inferred components for the peaks: peak #1: 7:C23; #2: C23; #3: 7,11:C25; #4: 5:C25; #5: 7,11:C27; #6: 5,9:C27 (+2-methylhexacosane).

(Figure 2(a)). The result showed that even by the no-choice experiment, the number of matings occurred within 1 hour between TW1 females and Mel6 males remained to be very small compared to that between Mel6 females and males (Figure 2(c)).

The low frequency mating between TW1 and Mel6 could be due to either male refusal or female rejection. In order to ask if there is Mel6 male refusal of TW1 female, we conducted the no-choice experiment by Mel6 female perfumed with cuticular hydrocarbons of TW1 female. Cuticular hydrocarbons can be transferred to other females by crowding the flies with the donor flies [14]. In order to see if cuticular hydrocarbons of TW1 have an inhibitory effect on mating, they were transferred to Mel6 females before conducting no-choice experiment. The experiment showed that Mel6 females coated with cuticular hydrocarbons from TW1 females mated less frequently with Mel6 males compared to the control Mel6 females (Figure 2(d)). This indicated that cuticular hydrocarbons of TW1 females have an inhibitory effect on Mel6 male mating behavior.

We then compared the profiles of cuticular hydrocarbon components between the two strains by GC (Figure 3). The identities of the corresponding cuticular hydrocarbon components of the peaks were inferred from the previous studies [28, 29]. The peaks, which showed qualitative differences (presence/absence or extreme high/low) between the two strains, were marked by numbers in Figure 3. Their relative quantities (% area of the peaks) are shown in Table 1. Inseparable peaks and peaks with less than 2% of the whole peak area were not included in the comparison.

Mel6 females had a relatively simple content of cuticular hydrocarbons with almost no trace of peaks in the C23 group (around peaks #1 and #2), whereas the overall complexity of the TW1 female profile was similar to the typical wild type strains of *D. melanogaster* [12, 25]. The cuticular hydrocarbon profile of Mel6 females resembled that of Tai Y strain originated also from West Africa (Ivory Coast) with only traces of C23 hydrocarbons [12, 25].

The peaks #5 and #6 were inferred to be 7,11:C27 and 5,9:C27, respectively. However, 5,9:C27 peak is likely to be confounded with comigrating 2-methyl-hexacosane [24, 34]. The polymorphism in the ratio of these two C27 dienes has been well documented in the worldwide samples of *D. melanogaster* [24, 34, 35]. Although there is an association of *desat2* genotype, which is responsible for the polymorphism, with a sexual isolation between Zimbabwe and cosmopolitan populations of this species [36], this polymorphism does not seem to induce assortative mating pattern in general [34].

Next, we used two other types of females with different cuticular hydrocarbon profiles to test how mate preference of Mel6 males changes (Figure 4). Mel6 males did not discrim inate between Mel6 and BZ1 females, which showed similar cuticular hydrocarbon profiles to TW1 females in peaks #1–6 except a smaller peak in peak #3 (Figures 4(a) and 4(c), Table 1). We also tested F1 females from Mel6 × TW1 cross, which also showed a smaller peak in peak #3 compared to that of TW1 female, and intermediate height peaks of #5 and #6 (Figures 4(b) and 4(d), Table 1). Mel6 males clearly favored these F1 females over TW1 females (Figure 4(b)), which gave a mating pattern similar to Figure 2(b). Previously known polymorphism in the ratio of 7,11:C27 (peak #5) and 5,9:C27 (peak #6) does not seem to affect the mate preference of Mel6 males. Taken together, these comparisons are consistent with the notion that the component in the TW1 female cuticular hydrocarbon, which has an inhibitory effect on mating with Mel6 males, is in the peak #3. The component of this peak is likely to be a C25 diene and is inferred to be 7,11:C25 from the previous literatures [28, 29].

In order to ask how general is this mate discrimination of Mel6 males against TW1 females, we tested 2 other strains originated from the same collection point of Mel6 in West Africa (Mel7 and Mel8) and strains from Brazil (BZ1) and Tonga (VAV1) to investigate whether their males show nonrandom mate choice between Mel6 and TW1 females. In order to avoid the effect of other preference factors, the male Mel6 was substituted by the test strain male in the double-choice experiment identical to Figures 2(a) and 2(b). The results in Figure 5 indicated that males from two other strains originated from the same collection site as Mel6 in West Africa mated more with Mel6 females than with TW1 females (Figures 5(a) and 5(b)) giving the similar pattern as in Figure 2(b), whereas strains from Brazil (BZ1) and Tonga (VAV1) did not show a clear refusal of mating with TW1 females (Figures 5(c) and 5(d)). These patterns suggest that Mel6-type mate discrimination is endemic to individuals from its collection site in West Africa.

Table 1: Relative quantity (% area) of the GC peaks designated in Figure 3. Sample sizes are shown under the strain names. Mean ± S.D. Values are shown.

| Strain | Peak #1 7:C23[a] (%) | Peak #2 C23[a] (%) | Peak #3 7,11:C25[a] (%) | Peak #4 5:C25[a] (%) | Peak #5 7,11:C27[a] (%) | Peak #6 5,9:C27[a] (+2-methylhexacosane) (%) |
|---|---|---|---|---|---|---|
| TW1 ($n = 3$) | 2.65 ± 0.07 | 6.44 ± 0.11 | 10.96 ± 1.25 | 0.00 ± 0.00 | 46.77 ± 0.61 | 2.22 ± 0.08 |
| Mel6 ($n = 3$) | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 2.67 ± 0.05 | 7.54 ± 6.53 | 58.77 ± 5.18 |
| BZ1 ($n = 2$) | 2.46 ± 0.31 | 8.64 ± 0.99 | 3.95 ± 0.07 | 0.00 ± 0.00 | 52.11 ± 0.33 | 3.06 ± 0.42 |
| Mel6 × TW1 F1 ($n = 2$) | 0.00 ± 0.00 | 4.43 ± 0.09 | 1.85 ± 0.02 | 1.98 ± 0.06 | 25.60 ± 0.34 | 19.55 ± 0.30 |

[a] Cuticular hydrocarbon components inferred from the previous studies [28, 29].

## 4. Discussion

In order to analyze within species variations in mate preference, we first performed a survey of mating frequency between different strains of *D. melanogaster* and its sibling species, *D. simulans*. Crossability between these two species has been reported to be asymmetric; *D. melanogaster* females mate relatively easily with *D. simulans* males, but the reciprocal cross is more difficult [37, 38]. Our results did not follow this pattern (Figure 1). Not many *D. melanogaster* females mated with *D. simulans* males except those of TW1 (Figure 1(a)). In the reciprocal cross, in addition to S2 that has been documented as a highly crossable strain to *D. melanogaster* males [33], there was another strain W86 from Madagascar that mated well with *D. simulans* males (Figure 1(b)). Our results suggest that the asymmetry in crossability may depend highly on the strains tested.

What was more notable in our survey was that the crossability between the two sibling species was highly variable among strains (Figure 1), which indicated that the signals presented by either or both sexes and the perception and response to those signals are not completely fixed within each species. These intraspecific variations may have become more apparent in our survey using inbred strains (except S2), because some of the recessive alleles that affect those traits may have become homozygous during the inbreeding process. This inbreeding effect may have affected the asymmetry in interspecific crossability to some extent as well.

Because of the differential mating frequencies of TW1 and Mel6 against *D. simulans* strains (Figure 1), we predicted that their differences in courtship signals and perceptions may cause partial incompatibility between these strains. As predicted, low mating frequency between TW1 females and Mel6 males was observed (Figures 2(b) and 2(c)). Mel6 males were choosy in both interspecific and intraspecific crosses. The factors responsible for the low mating frequency between TW1 females and Mel6 males turned out to be a difference in cuticular hydrocarbon component and its perception. Since C25 dienes are not reported in the cuticular hydrocarbons of *D. simulans* females [12], different factors may be responsible for the interspecific mating frequency differences between these two strains. It is certainly of our interest to identify the factors involved in differential mate preferences against interspecific individuals.

There are several cases of partial incompatibility in mating between strains or populations of *D. melanogaster*. One is between populations from Zimbabwe (Z) and cosmopolitan (M) areas, in which case there is a strong behavior incompatibility between Z females and M males [7]. TW1 female-Mel6 male incompatibility is not part of this Z-M system, because the compatibility in the former is between a non-African female and an African male, which is opposite of the latter. Similarly, US and Caribbean populations exhibit partial incompatibility [10, 11] and there are also cases reported in Brazzaville, Congo [8] and at the "Evolution Canyon" in Israel [9] where two closely located populations show certain amount of premating isolation. To determine whether our observation of mating incompatibility between TW1 and Mel6 is another case of interpopulational sexual isolation or not, we should await a larger survey of strains, especially since we currently have found only one TW1 type strain. However, our study demonstrates that a single cue, a particular cuticular hydrocarbon blend, could produce differential responses of males from different strains within *D. melanogaster*.

The cuticular hydrocarbon transfer experiment showed that it is not the female rejection, but the male refusal of females, which brings about the reduced mating frequency between TW1 females and Mel6 males (Figure 2(d)). It is generally thought that females drive sexual isolation in *Drosophila*, because males are observed to court females largely indiscriminately while females seem to be able to effectively reject unpreferable males. However, our experiment clearly indicated that, in some cases, males actually do choose females according to the cuticular hydrocarbon profiles of females. This is not surprising since it is known that the female cuticular hydrocarbons contain components that exhibit both stimulatory and inhibitory effect on mating [16, 23, 24]. A known inhibitory sex pheromone for *D. melanogaster* males, 7-tricosene, is predominant in male cuticular hydrocarbon and perceived as a bitter stimulus for the males [19]. This molecule is at a normal quantity in TW1 females (peak #1, Table 1). Our interpretation that cuticular hydrocarbons of TW1 contain inhibitory factors instead of lacking stimulatory components comes from the fact that the only detectable peaks that had higher quantity in Mel6 were peaks #4 and #6 (Figure 3) and that both peaks were not present in BZ1 females that mated well with Mel6 males (Figure 4(a), Table 1). However, a subtle balance between the quantities of excitatory and inhibitory components may be critical for the decision making by males. Future assays using purified hydrocarbons should elucidate the combined effects of different components.

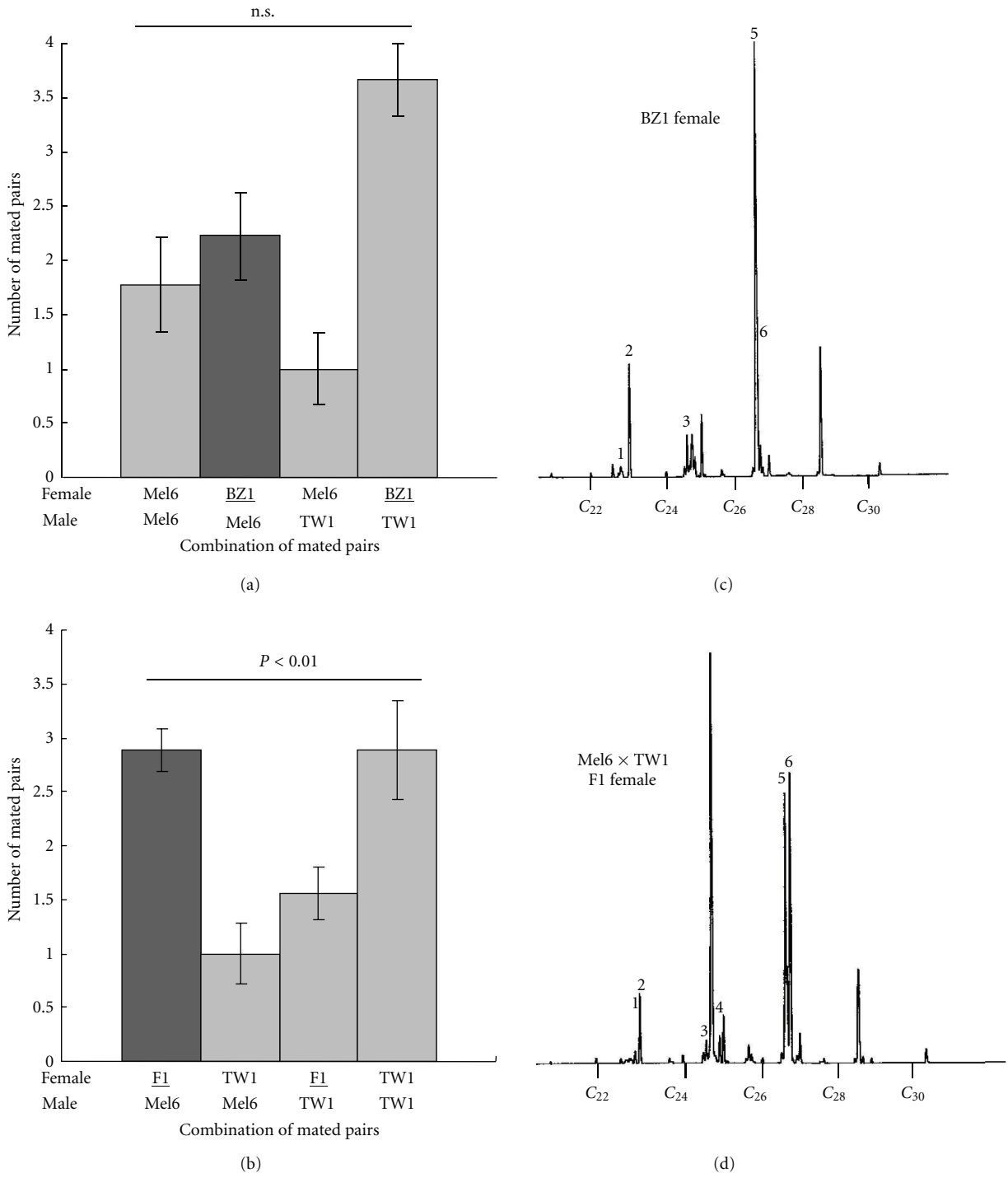


Figure 4: Mate discrimination by Mel6 males towards females with different cuticular hydrocarbon profiles. (a) Number of mated pairs in the double-choice mating experiment by substituting Mel6 females with BZ1 females in Figure 2(b). The pattern did not deviate from random mating (Cochran-Mantel-Haenszel exact test $P > 0.10$). (b) Number of mated pairs in the double-choice mating experiment by substituting TW1 females with F1 females (Mel6 × TW1) in Figure 2(b). The pattern deviated from random mating (Cochran-Mantel-Haenszel exact test $P < 0.01$). (c), (d) Gas chromatograms of *n*-hexane extracts of BZ1 females and F1 females (Mel6 × TW1), respectively. The numbers indicate corresponding peaks in Figure 3.

Under the assumption that cuticular hydrocarbon blend from TW1 female has inhibitory effect on mating in Mel6 males, we identified a candidate cuticular hydrocarbon component, a C25 diene, that has a deterrent effect on Mel6 males. The component is inferred to be 7,11:C25 from the previous literature [28, 29]. A large increase in 7,11:C25 (+13.2% of the total hydrocarbon amount) was reported as a consequence of an RNAi knockdown of an elongase

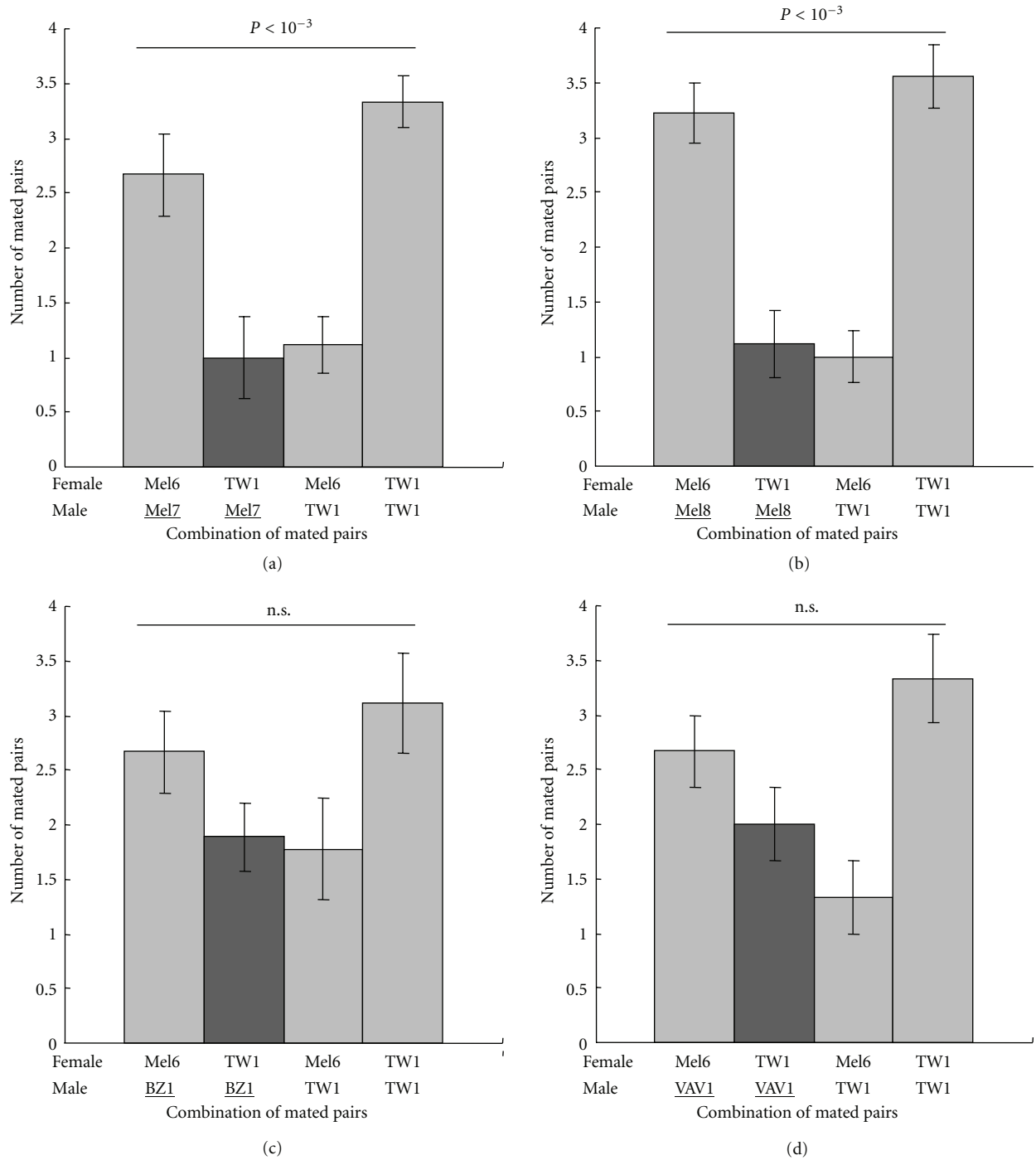


Figure 5: Mate discrimination by males from different strains. (a) Number of mated pairs in the double-choice mating experiment by substituting Mel6 males with Mel7 males in Figure 2(b). (b) Number of mated pairs in the double-choice mating experiment by substituting Mel6 males with Mel8 males in Figure 2(b). (c) Number of mated pairs in the double-choice mating experiment by substituting Mel6 males with BZ1 males in Figure 2(b). (d) Number of mated pairs in the double-choice mating experiment by substituting Mel6 males with VAV1 males in Figure 2(b). The substituted strain is indicated by an underline. The result of Cochran-Mantel-Haenszel exact test after figure-wide Bonferroni correction for multiple tests is shown for each experiment. Note that Mel7 and Mel8 strains are from the same collection site as Mel6.

gene, *eloF*, in *D. melanogaster* female [39]. Interestingly, those females showed decreased attractivity to Canton-S males. This observation is consistent with our conclusion that 7,11:C25 may serve as an inhibitory effect on the mate recognition of males. We should note that *eloF* knockdown females also showed marked decrease in 7,11:C29, which was not apparent in TW1 females (Figure 3).

Males from 3 strains originated from Benin (West Africa), Mel6, Mel7, and Mel8, did not readily mate with TW1 females, whereas 2 other strains from outside Africa

showed no such discrimination (Figures 2 and 5). The cuticular hydrocarbon profiles of Mel6 (Figure 3), and another isofemale line from West Africa, Tai Y, were similar with only traces of C23 hydrocarbons [12, 25]. Females of Mel7 showed a similar profile to that of Mel6 as well (data not shown). These observations suggest that *D. melanogaster* in regions of West Africa may have different sensitivity or response to chemicals on the female cuticles. There are some identified olfactory and gustatory receptors mediating male courtship behavior in response to sex pheromones [40–44]. The differential expression of these receptors among males from different strains could be a cause of the differential mate choice.

Courtship behavior is affected by conditioning prior to courtship; unsuccessful courtship reduces subsequent courtship in males [45, 46]. It has been shown that flies can have memory of at least one cuticular hydrocarbon component, *cis*-vaccenyl acetate, during the conditioning [47]. In our experiments, males were kept in groups prior to the mating experiments, which may have affected the memories of unsuccessful male-male courtship. However, an elevated level of the C25 diene was not observed in Mel6 nor TW1 males (data not shown), which suggests that there was no differential learning associated with the candidate inhibitory factor.

Our study was the first case to suggest that a C25 diene produced by a wild-derived strain has an inhibitory effect on mating behavior of males and to show that the sensitivity to this chemical varies among strains. Such modification in sensitivity could become a potential isolation factor during incipient speciation. Further investigation of the precise mechanisms and genetics underlying these intraspecific variations may help entangle the evolution of complex intersexual communication and mating behavior in flies.

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*Review Article*

# Evolutionary Implications of Mechanistic Models of TE-Mediated Hybrid Incompatibility

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New models of TE repression in plants (specifically *Arabidopsis*) have suggested specific mechanisms by which TE misregulation in hybrids might result in the expression of hybrid inviability. If true, these models suggest as yet undescribed consequences for (1) mechanistic connections between hybrid problems expressed at different postzygotic stages (e.g., inviability versus sterility), (2) the predicted strength, stage, and direction of isolation between diverging lineages that differ in TE activity, and (3) the association between species attributes that influence TE dynamics (e.g., mode of reproduction, geographical structure) and the rate at which they could accumulate incompatibilities. In this paper, we explore these implications and outline future empirical directions for generating data necessary to evaluate them.

## 1. Introduction

In many plants and animals, hybrid inviability (lethality) or hybrid sterility act as postfertilization barriers to hybridization [1]. These incompatibilities can be explained by negative genetic interactions between two or more loci under the Dobzhansky-Muller model of hybrid incompatibility [2, 3]. The Dobzhansky-Muller model does not specify the nature of the genetic elements that can lead to hybrid incompatibilities, and to date there are few cases where the loci responsible have been directly identified [4, 5]. The idea that transposable elements (TEs) can influence hybrid sterility is well established in *Drosophila* and has been highlighted in studies of intraspecific crosses resulting in hybrid dysgenesis [6–8]. However, since TE mobilization in hybrids between animal species is less frequently observed than in plants [9–11], skepticism has arisen surrounding the role that TE movement plays in the evolution of reproductive isolation [1, 11]. Recent epigenetic studies in plants, however, are providing evidence that a common genetic pathway, involving siRNA regulation of transposable elements, might lead to both hybrid inviability and hybrid sterility [12–14]. In this paper, our goal is to examine implications of these mechanistic models for the genetics and evolution of reproductive isolation due to TE misregulation.

Transposable element suppression in somatic cells is generally conserved among all organisms and requires three steps [15], see Figure 1. (1) Transposon transcripts are detected by complementary small RNAs derived from previously transcribed transposons (typically siRNAs in plants and piRNAs in animals). (2) These transcripts are posttranscriptionally cleaved by small RNA-protein complexes creating small RNAs that are amplified through an RNA-dependent RNA polymerase. (3) These newly derived small RNAs are used to target transposon transcripts as in step (1) or to target the transposon sequences in the genome to induce DNA methylation and repressive chromatin modifications. Slight modifications to the pathway occur during regulation of TEs in germ line cells.

Small RNA pathways have been proposed to play a role in regulating imprinted genes and genome-wide methylation patterns; this role may influence gene expression in hybrid individuals. In this review, however, we will focus on TE regulation during the development of male and female plant gametophytes, and the consequences this could have for the expression of postzygotic hybrid incompatibilities. First, we

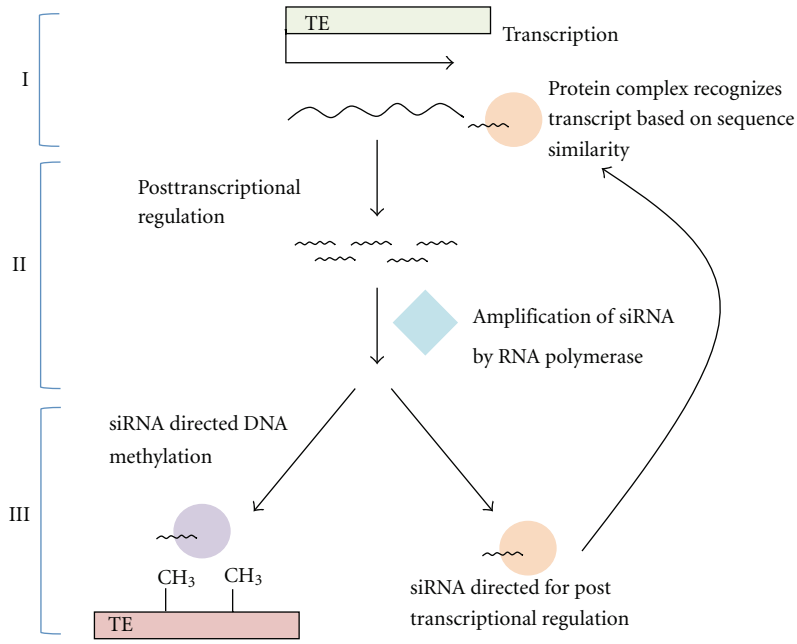


Figure 1: A general model of the siRNA pathway demonstrating the three processes observed in both plants and animals. (1) Transposon transcripts are detected by complementary small RNAs derived from previously transcribed transposons. (2) These transcripts are post-transcriptionally cleaved by small RNA-protein complexes creating small RNAs that are amplified through an RNA-dependent RNA polymerase. (3) These newly derived small RNAs are used to target transposon transcripts as in step (1) or target the transposon sequences in the genome to induce DNA methylation and repressive chromatin modifications.

briefly describe the current mechanistic models of TE regulation in gametogenesis, including prior connections that have been drawn between TE misregulation and the expression of hybrid incompatibilities, and indirect evidence for this association in plants (i.e., *Arabidopsis*). Second, we discuss unexplored consequences of these emerging models for the genetics and evolution of species barriers based on this mechanism. Based on this, we identify several implications of evolutionary importance: (1) TE-misregulation is a mechanism that can produce nonindependent accumulation of hybrid problems at different developmental stages of isolation between species (e.g., hybrid inviability and hybrid sterility). (2) Where this mechanism is an important contributor to reproductive isolation, the direction and strength of hybrid problems should be predictable based on lineage differences in TE abundance ("load") and sequence identity. (3) Factors influencing the divergence of TEs among lineages should also influence when and where TE-mediated hybrid incompatibility is most likely to be observed. We identify empirical data that will be essential to assess these inferences in the future, and potential approaches for generating these data. Finally, we sketch some similarities and differences between plants and animals that could affect hybrid problems related to TEs in these groups. One of our goals is to anticipate patterns of reproductive isolation that might operate at different stages or under different ecological or evolutionary scenarios, if TE misregulation (as described by these mechanistic models) is an important contributor to reproductive isolation.

## 2. Mechanistic Models of TE Suppression during Male and Female Gametogenesis

Recent findings have prompted Slotkin et al. [16] to propose a companion cell model for TE regulation in male (pollen) gametogenesis that is similar to a model of piRNA accumulation in *Drosophila* egg development [17]. In angiosperm male gametes, the growing (postpollination) pollen tube contains three nuclei; two are sperm nuclei that will be involved in fertilization (see below), while one is a vegetative nucleus that does not fuse with the maternal gametophyte [18]. In the Slotkin et al. [16] model, the vegetative nucleus of pollen acts as a companion cell that "sacrifices" itself so that the sperm cells can maintain correct TE suppression. In this nucleus, TEs are demethylated, increasing transcription of elements (most that are normally somatically silenced). These transcripts are posttranscriptionally processed, creating a pool of small RNAs. These small RNAs are transported to the sperm nuclei where they direct methylation of TEs, effectively "resetting" the appropriate suppression of TEs in the germline nuclei prior to fertilization. The transcription of TEs in the pollen vegetative nucleus is initiated after the loss of heterochromatin and MET1 (cytosine-DNA-methyltransferase) and DDM1 (decreased DNA methylation) proteins [16], indicating an actively regulated process that is specific to this nucleus only. The small RNAs produced in pollen are predominantly 21-nt long and, besides translocation to the sperm cell, these small RNAs are also delivered into the zygote and endosperm [16], leading to potential

consequences for regulation, and misregulation, at postfertilization stages.

Similar to pollen development, the TE-derived small RNAs necessary for TE regulation in the female germ line (gametophyte) are created in an adjoining cell that will not contribute DNA to the next generation (in this case, epidermal cells lining the ovary) [19]. In angiosperms, the mature female gametophyte is composed of seven cells containing eight genetically identical haploid nuclei (all the products of a single initial meiotic product), surrounded by maternal (sporophytic) tissue. Two of these cells will contribute tissues to the F1 following typical "double fertilization": the haploid ovule (egg) fuses with one sperm cell to give rise to the developing embryo; the doubled-haploid ($2 \times 1N$) "central cell" fuses with the second sperm cell to give rise to the 3N endosperm—a nutritive tissue that accumulates resources postfertilization to support development of the embryo. During development of the female gametophtye, small RNAs are translocated from the "sacrificial" epidermal cells into the ovule and central cell; these act to silence TEs in the developing embryo and endosperm after fertilization [20]. Another specific component of TE regulation, the Argonaut protein AGO9, is also expressed in the maternal sporophytic cells, but not in germ cells, and interacts preferentially with the TE-derived 24-nt small RNAs to suppress TE activity in the female gametophyte [19].

## 3. Connections between TE Misregulation and Hybrid Incompatibility

Based on these emerging mechanistic details, several models have recently been proposed connecting TE regulation and hybrid failure. These models have focused primarily on seed failure via misregulation of development in the endosperm [12, 13, 21], partly because some of the most evident hybrid incompatibility phenotypes in *Arabidopsis* involve endosperm failure in interspecies and interploidy crosses. Both types of crosses give rise to seed collapse (inviability) due to either overproliferation and failure to cellularize, or under-proliferation and premature cellularization, in the hybrid endosperm [22–24]. Because these phenotypes are reminiscent of the examples of overgrowth in *Peromyscus* [25, 26], earlier models hypothesized that hybrid failure results from misregulation of imprinted genes. Recently, however, Martienssen [13] proposed an alternative model where mismatches in small RNAs contributed by each parent are responsible for endosperm failure, based on the evidence that TE sequences and TE abundance differ substantially between the *A. arenosa* and *A. thaliana* genomes [21, 27]. Martienssen [13] suggests that if TE sequences in the central cell do not match siRNA in the pollen or, reciprocally, if siRNA from the female germ line do not match TE from the sperm, then TEs will be active in the endosperm causing endosperm failure. Martienssen's model does not specify whether small RNAs have to match in terms of sequence similarity or overall quantity, as the crosses on which his model is based (*A. thaliana* by *A. arenosa*) potentially involve both differences in quantity and sequence.

The idea of an interaction between male and female "factors" controlling endosperm development has been used previously in the *Arabidopsis* literature. For example, Josefsson et al. [21] proposed a model—"dosage-dependent induction"—to explain endosperm failure in terms of the interactions between maternal and paternal factors that they observed in *Arabidopsis* hybrids, though they did not implicate TE-derived small RNAs. In their model, it is the sole responsibility of the maternal parent to deliver the proper number of "repressors" to saturate target sites in the maternal genome as well as target sites contributed by the paternal genome. If the total amount of repressor is insufficient, both maternal and paternal target sites will escape silencing. Conversely, extra doses of maternal genome can "rescue" an otherwise incompatible cross by increasing the total number of repressors. This model is generally consistent with longstanding observations in numerous species crosses, where simply manipulating maternal dosage can alter the compatibility of a cross (e.g., [28, 29]). The model is fundamentally different from the Martienssen [13] model because the male only contributes targets that require silencing but is not able to silence TEs that it contributes or that the maternal parent contributes. Michalak [12] has reinterpreted the "dosage-dependent induction" model in terms of small RNAs. In his example, he assumes that if there is a difference in the deposition of maternally loaded small RNAs between species, or if these small RNAs differ in their capabilities to suppress their targets, TEs can become active in hybrid crosses. The activation of TEs can cause endosperm failure, thereby acting as a reproductive barrier.

In both models, hybrid incompatibility is a property of TEs being preferentially activated in the endosperm, with no focus on TE activation in the embryo. One compelling reason that TE activation might preferentially occur in the endosperm is the empirical observation that the endosperm is loaded with RNAs by both parents [13], and gene regulation in the endosperm is, therefore, potentially influenced by both parental genomes. The embryo, in contrast, does not play a large role in early seed development and is less influenced by parental provisioning as most gene expression beyond the first few cell divisions is regulated within the embryo itself [30].

Both models provide new testable predictions and have the potential to provide a mechanism for earlier models of endosperm failure (e.g., the "endosperm balance number" model; [28]), but these models currently have a limited scope as general explanations of hybrid failure between species. In particular, they do not address expectations or predictions with respect to (a) the expression of hybrid failure at several other stages of reproductive isolation; (b) the differential effects of lineage differences in TE/small RNA abundance ("load") versus TE/small RNA sequence identity; (c) the evolutionary/ecological factors that can influence TE dynamics, and therefore the expected accumulation of isolation between linages that are differentially affected by these factors. Here, we address these different factors and some of their implications for the evolution of isolating barriers.

*3.1. Broader Applications: TE-Mediated Hybrid Incompatibility at Other Isolation Stages.* Although current plant models have focused on seed failure (endosperm breakdown in early F1 seed), there is reason to believe that inappropriate TE mobilization could also influence other isolation stages, including F1 male and female sterility. Indeed, the two models we have discussed are very similar to the piRNA models of transposon silencing in animals because they assume that TEs are reactivated in specific accessory cells to enhance small RNA-triggered silencing of TEs in germ line cells [20]. The phenotype observed in *Drosophila* studies of TE-mediated "hybrid dysgenesis" is male sterility. Hybrid sterility (in the form of pollen sterility) has also been observed in crosses between diploid *Arabidopsis* species [31], and Martienssen [13] discusses how the dysgenesis model could also be applied to pollen sterility in *Arabidopsis*. In addition to F1 hybrid male sterility, hybrid female sterility is also a potential consequence of TE-misregulation in a hybrid genome. For example, in the female gametophyte, proper regulation of TEs has been associated with restriction of cell fate [19]; if TEs are not correctly suppressed in the megaspore mother cell, aberrant phenotypes (including two female gametophytes in a single ovule) can occur, leading to female sterility. Finally, mutant studies indicate that misregulation of small RNAs has the potential to prevent proper double fertilization [32], although this empirical example does not involve TE-derived small RNAs.

These examples suggest that TE-mediated hybrid incompatibilities could occur at a range of stages, from early F1 inviability to F1 sterility. Given this, are there consequences for our understanding of the evolution of hybrid incompatibilities? TE-based models suggest a direct mechanistic connection, and therefore nonindependence, between different stages of postzygotic isolation in a single cross. This is inconsistent with most current models of the evolution of hybrid incompatibility, which assume independence among fixations contributing to different stages of isolation [33]. This independence assumption is reasonable for many loci contributing to species barriers, whose hybrid incompatibility effects are thought to be incidental by-products of evolutionary divergence at loci from many different potential developmental or reproductive processes [1]. However, the tight mechanistic connection between regulation of TEs during different developmental stages indicates that this independence assumption is likely violated, and therefore that predictions about hybrid incompatibilities that rely on this assumption might not hold for TE-mediated hybrid incompatibility. For example, under the Dobzhansky-Muller model of hybrid incompatibilities, the number of reproductive isolation loci is predicted [34] and observed [35, 36] to "snowball" (i.e., increase faster than linearly with time) between diverging lineages. The snowball prediction emerges from the condition that each new fixation within a diverging lineage can potentially interact with every other evolutionary change that has preceeded it during lineage divergence [33, 34]). When fixations producing reproductive isolation instead involve interactions between a limited, nonindependent set of loci, these predictions do not hold (e.g., [37]). We suggest, then, that TE-mediated incompatibilities might not follow the predictions of such models. Instead, TE-mediated incompatibilities might behave similarly to other "conflict" driven hybrid incompatibilities [4], where reproductive isolation is due to interactions between a small subset of loci evolving according to antagonistic coevolutionary processes (see further below).

*3.2. Differentiating the Contribution of Divergence in TE/Small RNA Quantity versus Sequence Identity.* Current models of hybrid failure are coy about the range of mechanisms by which maternal/paternal "mismatch" in TE regulation can occur. In the *Arabidopsis* interspecific crosses, it is clear that the abundance of TEs—either TE copy number or size of the resulting small RNA pool contributed by the paternal and maternal genomes—could determine the outcome of the cross, as both are correlated with endosperm failure. In this case, stoichiometric mismatch is responsible for the inappropriate regulation of either gametogenesis or postfertilization development (Figure 2(a)). Alternatively, parental "mismatch" could be due to sequence divergence between TEs in different lineages, whereby small RNAs generated from one lineage may fail to recognize or to effectively interact with target sequences from the alternative lineage, due to base-pairing mismatches (Figure 2(b)). Although there is no direct empirical evidence, it has been suggested that sequence specificity plays an important role in TE suppression [38, 39] and the proteins that interact with small RNAs rely on sequence complementarity to target TEs for methylation and silencing [40]. Given this, transcripts from one TE copy may not be able to target slightly different TE copies, although currently it is not clear how much sequence divergence must occur before TE copies can no longer suppress one another.

Regardless, determining the relative effect of TE abundance ("load") versus sequence divergence on hybrid incompatibilities might be important because, as we argue here, these two different forms of divergence are expected to be more influential at different stages of reproductive isolation. These expectations can be inferred from the current mechanistic models of TE suppression at different developmental stages in plants. For example, TE silencing during prefertilization gametogenesis and postfertilization endosperm development both rely on sequence complementarity between small RNAs and target sites [38, 39]. However, these two developmental stages differ in that silencing in the endosperm is dependent on parentally derived small RNAs whereas silencing during gametogenesis is regulated by self-generated small RNAs. This difference in the source of siRNA regulators creates the potential for different kinds of TE divergence to differentially affect early F1 viability following heterospecific fertilization, versus gametogenesis in an F1 hybrid individual and/or hybrid problems in later generation (e.g., F2) individuals.

First, consider the F1 offspring from an interspecific (hybrid) cross. In the developing hybrid endosperm, small RNA sequences from the maternal/paternal genome will not necessarily match target sites in the other (heterospecific) genome, but they will be capable of silencing TEs derived from their own (homospecific) genome. Some of this TE

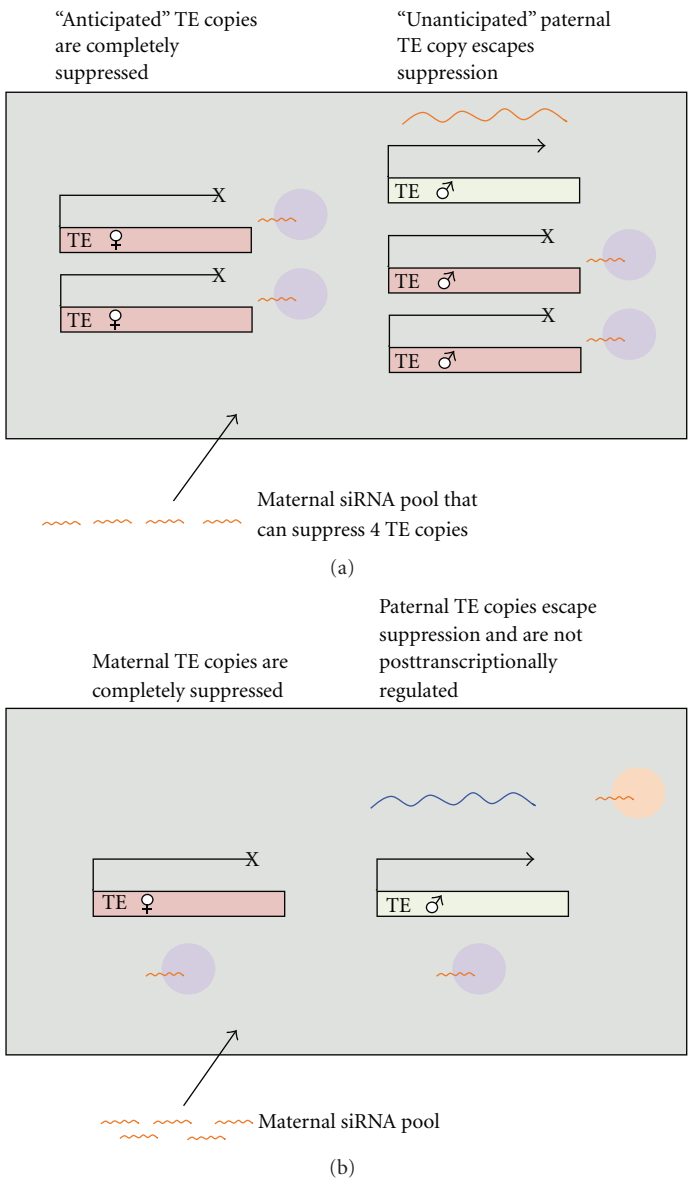


Figure 2: The misregulation of TEs due to a mismatch of maternal siRNA and paternal TE copies, consistent with the Martienssen and Josefsson models of endosperm failure. (a) TE load differences between parents: the maternal siRNA cannot suppress paternal TE copies due to excess of paternal TE copies. (b) TE sequence mismatch between parents: the maternal siRNA cannot suppress paternal TE copies due to differences in sequences of the TEs.

suppression takes place prior to fertilization, that is, in the sperm cells within pollen and in the female gametophyte (see above). Other regulatory control relies on postfertilization suppression, where it appears that the primary (but likely not sole; [16]) determinant of control is maternally loaded siRNAs. This "asymmetry" in control is important for the expression of hybrid problems in the early developing offspring. In particular, if the maternal parent contributes a quantity of small RNAs sufficient to regulate TEs in its own (diploid) genome, but not in excess to accommodate extra "unanticipated"copies from the other genome, TE suppression in the postfertilization offspring will be incomplete. This specifically occurs when a "low load" lineage acts as the maternal parent in a cross with males from a "high load" lineage. Large differences in TE copies among parental genotypes could also be magnified during the production of small RNAs, if this is nonlinearly related to TE copy number.

Note that differences in sequence identity might exacerbate these load differences; when either parent is lacking copies of a specific TE, the endosperm might be overwhelmed with TE activity. Again, the expectation is that this will be observed when "naïve" lineages act as the maternal parent in crosses to males from lineages that contain the novel TE, but not necessarily in the reciprocal cross. Regardless, it appears that differences specifically in TE load are most likely to be influence misregulation of TE suppression in early F1

(e.g., endosperm) development. Note that details of this prediction rely on the supremacy of maternally loaded siRNAs during early offspring development; if paternally loaded siRNAs are as important as maternally loaded siRNAs, some expectations might not hold. For example, in the Martienssen [13] model, either parental genome can be "overwhelmed" whereas in the Josefsson et al. [21] model only the maternal genome has the potential to be overwhelmed.

Second, consider the fertility of F1 offspring from an interspecific (hybrid) cross. For TEs to cause hybrid sterility, they must be improperly regulated during gametogenesis in the hybrid individual. This might occur if the small RNAs created during gametogenesis cannot fully silence TEs inherited from both parents. F1 hybrids will have all possible target sites and small RNA-producing loci, so how could incomplete suppression occur in F1s? One possible circumstance is if siRNA-mediated TE suppression is haploinsufficient (either due to siRNA production or TE targeting), for example, if there is a nonlinear relationship between siRNA suppression efficacy and TE copy number. Note that this is more likely to be due to differences in TE sequence identity among parental lineages; haploinsufficiency applies only to those loci for which the F1 is functionally haploid (i.e., loci missing in one parental lineage). In comparison, differences in load may not be important during hybrid gametogenesis because F1 individuals would have a haploid complement of TE copies from both parents; thus there are no "unanticipated" TE copies to be accounted for, unlike during endosperm TE suppression. Regardless, the expression of F1 sterility due to divergence in TE identity is expected to be "symmetric;" reciprocal F1s should show the same sterility effects.

Finally, consider the viability and fertility of recombinant later generation (e.g., F2) hybrids. In addition to the above effects, in these individuals the potentially independent segregation of TEs and their regulators is important. In animals, small RNAs used for TE suppression are primarily derived from TE clusters located in (sometimes distant) heterochromatic regions [41]. TEs in these clusters are generally no longer active and as a result their DNA sequence can change rapidly. If the hybrid progeny does not inherit these clusters (even if they have inherited other TE copies), or if sequences in the clusters no longer match the active copies outside of the cluster, then they might not generate sufficient small RNAs. If the small RNA cluster model is not appropriate for plants, variation in the efficiency of producing small RNAs may still exist between TE copies and could influence the production of small RNA pools.

Overall, on the basis of these mechanistic models, we infer that TE "load" (copy number) differences are likely more important at early (embryonic) stages of hybrid formation, when parentally (especially maternally) loaded siRNAs are critical for TE regulation. In comparison, differences in TE identity are likely more important for the expression of hybrid sterility. In addition, the symmetry of isolation between reciprocal crosses is expected to differ between these stages. Given this, depending on the factors that differentially affect these modes of TE divergence, different expression of TE-mediated hybrid incompatibility might be expected under different ecological and evolutionary scenarios.

*3.3. Evolutionary Expectations from Predicted TE Dynamics.* Evolutionary models have already been used to describe and predict TE dynamics within and between populations. Understanding the forces affecting TE outbreaks, proliferation, spread, and suppression might therefore help in predicting conditions under which lineages can differ in TEs, and therefore when TEs are most likely be involved in species barriers between them.

*3.3.1. Factors Influencing Evolutionary Divergence in TE Abundance and Identity.* For TE misregulation to influence the expression of hybrid incompatibilities, diverging lineages must differ in the activity and/or identity of their TEs. What conditions influence the accumulation of different TE copy number (load) or novel TE sequences among lineages? TE dynamics have been examined extensively using population genetic models, especially to understand conditions that produce a stable transposition-selection balance [42]. Some models have incorporated host responses in the form of alleles that suppress activity of new TE invaders [43], although we are aware of only one model that specifically incorporates the possibility of sRNA-mediated TE suppression [44]. Drawing from these models, Blumenstiel [45] has summarized the four phases of TE invasion in host populations (and the mechanisms/factors that influence these four phases): (i) invasion of a new TE (either via mutation of an existing TE, or via horizontal transmission); (ii) TE proliferation, polymorphism, and fixation in the host population (where the rate and extent of proliferation depends on transposition rates, selection against the negative effects of TEs in hosts, migration rates in the host species, and drift); (iii) the origin of a repressor locus (via a new insertion (mutation) with repressive effects); (iv) fixation of the repressor allele, and decay of the new TE family. The completion of phase IV can depend on the degree of linkage between the repressor and its targets, and whether there are other collateral effects of silencing on host function; under some conditions, the repressor allele is not expected to fix (see [45], and references therein).

Perhaps unsurprisingly, then, the factors affecting population differences in the proliferation and/or divergence of TEs are the standard evolutionary forces of mutation, migration, drift, and selection. Clearly, however, the relative influence of these factors could be affected by whether hosts can respond to new TE outbreaks by fine-tuning existing sRNA mechanisms of control [45]. For example, the (mutational) origin of a new suppressor allele becomes less important if hosts have a preexisting mechanism for suppressing the negative effects of novel TEs they encounter. Preexisting small RNA pathways might also influence the strength of selection against potential deleterious effects of TE proliferation. Indeed, models indicate that TE insertions that are the target of RNA-mediated silencing are more likely to drift to higher frequency (because their deleterious effects are moderated via this host control) than insertions that are not targets of RNA-mediated silencing [44].

Notwithstanding such observations, some factors seem more predictably associated with TE proliferation and spread regardless of, for example, the mechanism of TE control. In particular, migration can directly influence the rate at which TEs spread between populations. When there is gene flow, populations with different TE copy number can become homogenized because TEs have the potential to invade "empty" populations. In highly structured populations, however, the homogenization process is slow; as a result, heterogeneous TE copy number distributions (and, presumably, TE identities) can be maintained over long periods of time when TE proliferation is different between populations [46].

Migration or population subdivision will be influenced by life history and demographic characteristics of the host species. For example, on average, species with active dispersal mechanisms are expected (and observed; e.g., [47]) to be less subdivided than passively dispersing species. The magnitude of population subdivision can also be strongly influenced by the mode of reproduction. In plants, for example, selfing species are more strongly genetically subdivided than mixed maters or outcrossers (e.g., [47]). The mode of reproduction also influences effective population size (i.e., $N_e$ is smaller in selfing species) and therefore the relative influence of drift on TE dynamics. Drift can act to magnify population differences in TEs by allowing the stochastic accumulation of TE load [48, 49].

These observations suggest that TE accumulation might be expected to be greater among selfing lineages. However, some models indicate that the influence of mating system on TE copy number is dependent on the specific mode of selection acting against TEs [50, 51]: where selection acts on the deleterious effects of TE insertions, both the probability that a TE is lost when its initial frequency is low and the TE copy number increase as self-fertilization increases; when selection acts on the effects of ectopic recombination between TE copies, the exact opposite effects are observed. Interestingly, several studies that have compared TE insertion frequency in selfing and outcrossing lineages produce inconsistent results as to whether selfing lineages have lower or higher TE copy number [52–55]. Also, there is no clear evidence that TE copy number and self-fertilization rate are correlated [51]. This might suggest that the most pertinent factors for TE accumulation and differentiation are $N_e$ and migration, parameters that are only imperfectly correlated with mode of reproduction.

*3.3.2. Evolutionary Expectations about the Accumulation of TE Differences.* Given these complexities, based on the current models it is difficult to draw many strong generalizations about the factors that can influence the origin and spread of TEs, especially because it remains unclear how selection acts on TEs [56, 57], including those that are subject to siRNA-mediated repression [44] (and see below). Further work will be necessary to clarify this in the future. Nonetheless, it seems likely that factors that influence the frequency with which new TE outbreaks occur (such as low $N_e$ that reduces the efficiency with which TEs are suppressed) and the degree to which individual TE outbreaks are locally "quarantined" (such as the extent of population subdivision/migration) can influence the rates at which TE differences are able to build up between lineages. Similarly, factors that influence the rates of sequence evolution of TEs should also influence TE divergence. For example, antagonistic selection, including intergenomic conflict, has the potential to cause rapid divergence between populations [58]; thus conditions that contribute to the efficacy of antagonistic selection (such as the ability of parasites to escape from their own deleterious effects, via horizontal transmission) could also elevate the rates of lineage differentiation in TEs.

By influencing the accumulation of TE differences, such factors could influence the relative contribution of TE misregulation to the expression of species barriers among lineages. This is because these factors are not necessarily equally favorable for the fixation of other (non-TE) genetic differences that can also contribute to reproductive isolation. For example, as genomic parasites, TEs are able to "invade" empty populations (even when they have some fitness costs for their hosts), making quarantine fundamentally important in controlling TE homogenization. In comparison, complete population subdivision is not essential for adaptive genetic differentiation between populations, which can be more dependent upon the strength of local selection against immigrants. Similarly, conditions favoring antagonistic coevolution need not be the same as those favoring other adaptive fixations. Synergism among such factors might be especially favorable to TE differentiation. For example, under a geographic mosaic model, population subdivision and local (antagonistic) selection could jointly contribute to divergence in TE load and sequence identity [59, 60], although this is also true of any other potential isolating factors that are also subject to antagonistic coevolution. Regardless, given the historical attention to TE dynamics, it is surprisingly difficult to make strong predictions about factors expected to promote the accumulation of TEs between species. In future, perhaps the most clarity will not come from theoretical approaches, but rather from more taxonomically diverse and replicated empirical comparisons of TE differentiation between lineages that differ in key biological features.

*3.3.3. Possible Targets of Evolutionary Change and Causes of RI.* Even with limited clarity about evolutionary conditions favoring TE differences, there are some suggestive evolutionary targets that might be responsible for lineage differentiation in TE proliferation and control. For example, under the companion cell model, TEs must move from somatic cells to the germ cells to ensure they are passed to the next generation. It is at this stage of development that the interaction between TEs, suppressor alleles, and the small RNA pathway can be subject to strong selection [44] and therefore potentially rapid lineage divergence. Active TEs can only escape suppression if their sequence is divergent from small RNA producing loci or, if they produce small RNAs, when these RNAs do not interact efficiently with proteins in small RNA pathways. If small RNA pathways are under strong selection to suppress TEs in their genome, antagonistic selection from TEs trying to escape suppression can cause arms race dynamics. The piRNA machinery in some *Drosophila*

has been shown to be under strong positive selection, implying a potential arms race (but see [61]), although it has yet to be shown that selection is acting on TEs to escape suppression [62]. It is known in animals that Argonaut and Piwi proteins rely on sequence complementarity to direct TE methylation and suppression. If these proteins are fine-tuned to target specific TEs, then they may be less efficient at interacting with small RNAs from novel TEs and therefore at silencing these TEs.

Other potential targets of selective differentiation are host regulators of small RNA pools. For example, a general step in the small RNA directed TE-silencing pathway is amplification of small RNAs by an RNA-dependent polymerase. In *Arabidopsis*, the main polymerase involved in RNAi activities, including TE suppression, is Pol-IV [63]. As Pol-IV is not necessary for survival, selection acting on Pol-IV might be similar to forces affecting piRNA machinery in animals, including specialization for the TEs that occur in a specific genome; in this case, Pol-IV in hybrids may not be able to efficiently process novel small RNAs. Interestingly, changes in these targets might also result in more "standard" Dobzhansky-Muller type incompatibilities. RNA polymerases typically consist of 12 subunits, and specialization in different lineages might render subunits incompatible with their counterparts from different lineages when brought together in hybrids. In this scenario, incorrect enzyme assembly might disrupt protein function, similar to the description of PcG complex dynamics by Ishikawa and Kinoshita [64].

## 4. Future Empirical Approaches Linking TE Misregulation to Hybrid Incompatibility

To date, models inferring the involvement of TE misregulation in hybrid incompatibility are more suggestive than definitive, and unambiguous evidence connecting these phenomena has yet to be shown. Indeed, the specific molecular mechanisms by which TE derepression might cause cell death (and therefore hybrid problems) remains unknown, and there is currently no published evidence that mechanistically links inactivation of pathways that regulate TE suppression with hybrid sterility or lethality. In some cases, there is evidence that TE derepression has no significant fitness effect in hybrids. Chen et al. [27] have demonstrated that RNAi knockouts for met1 in *Arabidopsis suecica* do exhibit increased levels of TE expression but sterility is not observed in these individuals and the increase of TE expression is not seen in resynthesized allotetraploids. Similarly, maize lineages appear to have large differences in TE content and abundance [65] but crosses among them do not show evidence of deleterious incompatibility phenotypes (e.g., [66]). Indeed, it is unclear whether mobilization *per se* should be predominantly responsible for hybrid incompatibility phenotypes; for example, an alternative mechanism is that TEs indirectly affect incompatibility through the collateral misregulation of genes that have acquired TE regulatory elements or of genes adjacent to these loci [67]. Ultimately, these are questions that must be addressed if the aim is to truly assess the evolutionary importance of TE-mediated isolation mechanisms.

Given this lack of empirical data, to demonstrate that TE misregulation is responsible for hybrid incompatibilities in any given system, several pieces of empirical evidence will be essential. First, lineages must differ in TE and small RNA load and/or identity, and in the parental contributions of these to offspring; if they do not, divergence in TEs clearly cannot be responsible for hybrid problems. Second, lineage divergence in TE load or identity must be consistently associated with the phenotypic expression of postzygotic isolation, such as, reproductive and developmental problems in hybrids. Finally, the expression of specific reproductive isolating barriers must be functionally linked to TE derepression. Fortunately, in combination with classical genetics, several emerging empirical approaches now make these pieces of evidence attainable now or in the near future. These data will also be useful in evaluating some of the expectations we have identified above.

*4.1. Characterizing Lineage Differences in TEs and Parental Contributions of Small RNAs.* Traditionally, quantifying genotype differences in TEs required approaches such as "transposon display," where selective PCR produces a fingerprint of TE insertions for each genotype [68]. This approach can be used to roughly quantify relative copy number and describe differences between host lineages, for a known TE family [52, 53, 69, 70]. Increasingly, however, whole genome sequencing is being used to describe the entire suite of TEs within a given genome [71, 72]. This approach is not limited to known TE families and therefore can, in principle, detect TEs with novel sequences in addition to copy number differences between different genomes.

In addition to describing TE load and sequence differences between lineages, next generation deep sequencing of small RNA pools is also now routinely used to quantify tissue-specific small RNAs and changes in small RNA production in mutant lines [73–76]. An understanding of each parental contribution of both TE copy number (as a measure of load) and small RNAs would be necessary to examine the predictions of the Josefsson et. al. [21] and Martienssen [13] models. For example, the main prediction of the Martienssen model is that endosperm failure will occur when maternal and paternal small RNAs do not match the TE sequences contributed by the other parent. Similarly, in the Josefsson et al. [21] model, differences in maternal small RNA contributions will determine endosperm development. Deep sequencing of small RNA pools in pollen, female gametophyte, and endosperm will allow researchers to identify which small RNAs are contributed by each parent. Small RNAs could be mapped to genomic or EST sequences to determine the identity of small RNA producing loci in the maternal and paternal genomes [41, 77]. As assembly algorithms improve for short sequences, small RNAs could be assembled *de novo*. Copy number could be assayed using inverse PCR techniques or qPCR based on sequences uncovered by deep sequencing.

Next generation sequencing, therefore, provides the technology necessary for describing lineage and parental differences in TEs and small RNAs, and for comparing hybrid small RNA pools to equivalent parental pools. Nonetheless, some limitations remain. As genome sequencing technology

improves, sequencing small RNAs and whole genomes will become easier but alignment and assembly is still a limiting step for repetitive elements. In addition, quantification of small RNAs can give an estimate of the total size of the small RNA pool, but determining the quantity contributed by a single TE is not yet achievable. Moreover, if the TE family is young and different insertions have identical DNA sequences, quantifying and identifying small RNAs to a specific TE locus is not possible. In plants, it is thought that all TE copies contribute to small RNAs, but the model emerging in animals is that clusters of mostly nonactive TEs contribute to the small RNA pool to target active TEs [41]. These challenges will need to be tackled by technological and bioinformatic advances in the future.

*4.2. Demonstrating the Association between Isolating Barriers and TE Differentiation.* To date, an association between differences in TE/small RNAs and endosperm failure has only been noted in crosses between *A. thaliana* and *A. arenosa*. However, in these specific crosses, several potentially contributing factors are difficult to disentangle: these species differ in TE sequence divergence, TE abundance, and ploidy. To more directly associate differences in TE/small RNAs with the expression of postzygotic barriers, it would be preferable to exclude factors, such as ploidy, that might also contribute to isolation via effects unrelated to TE differences. One such approach would be to compare the expression of isolating barriers among groups of closely related (homoploid) species that differed in known TE load and/or sequence identity. Contrasting the mean strength, identity, and direction of reproductive isolation between TE divergent lineages with that between TE-similar lineages would provide a statistical assessment of the potential contribution of TE divergence (and misregulation) to the expression of species barriers and identify which isolation stages are differentially affected. These associations might also allow a finer resolution analysis as, depending on the species group, some species might only differ in TE copy number or sequence identity rather than differing in both. This would permit the effects of TE load and sequence identity to be evaluated independently. Finally, this approach would identify specific taxon pairs for which crossing evidence associating TE differences and isolating barriers is strongest. Such pairs could be targets for further detailed functional analysis.

A complementary strategy to understand the association between TE divergence and isolating barriers is to examine previously identified incompatibility QTL for the presence of TEs (or TE regulatory sequences). Fluorescent *in situ* hybridization (FISH) could be used to evaluate colocalization of TEs and incompatibility QTL, but this would rely on existing libraries of known TEs from the organism (or a closely related species) and *a priori* hypotheses of the importance of specific TEs. Positional cloning of QTL regions—itself a challenging empirical goal—could also facilitate examination of QTL for TE sequences.

*4.3. Evaluating Repression of TEs in Hybrids.* Finally, to unambiguously link reproductive isolating barriers with TE misregulation, it will be necessary to functionally connect specific developmental or reproductive problems in hybrids to TE derepression. To achieve this, a combined technique of methylation profiling and qPCR could be used to determine if TE transcripts (and specifically those known to differ between lineages) are active in the affected tissues. Methylation profiling can be achieved by using methyl-sensitive restriction enzymes on PCR products from DNA, but this requires a known TE sequence to develop appropriate primers. Bisulfite sequencing of DNA could provide an alternative approach, but a genomic DNA sequence is still needed to see which nucleotides have been converted after bisulfite treatment.

In this respect, profiling the endosperm appears to be the most straightforward initial step, as this tissue is relatively easily accessible via dissection from within the developing seed. For example, laser microdissection has already been used to specifically profile gene expression individually in the endosperm and embryo [78]; identical techniques could be used to isolate relevant tissues for siRNA and TE profiling. In comparison, to profile TE misregulation in the pollen, individual sperm cells must be dissected from their surrounding tissue, including the adjacent vegetative nucleus which is known to be demethylated (see above). Regardless, it is clear that established next generation and microdissection techniques can be used to address the specific question of whether divergent TEs are derepressed in developmentally abnormal hybrid tissues.

## 5. Conclusions

The idea that some reproductive isolating barriers (and therefore speciation processes) are the outcome of evolutionary conflicts has a long history but has been, until recently, mostly lacking in direct empirical support [4]. One such conflict is that between genomic parasites such as TEs and their hosts. Emerging mechanistic models of TE regulation via sRNA-mediated pathways provide renewed support for the hypothesis that TEs might be involved in the expression of interspecific reproductive barriers. Here, we have outlined several evolutionary corollaries that emerge from these mechanistic models, including the likely nonindependence of reproductive isolation acting at different stages, and the possible influence of particular demographic and life history factors on the relative susceptibility of lineages to hybrid problems based on TE misregulation. Some of these conditions themselves suggest further implications. For example, our inferences suggest that TE-mediated hybrid problems might be strongest at early stages of F1 development (e.g., during endosperm development) where lineage differences in TE load and identity can both contribute to hybrid problems. In comparison, F1 sterility is likely only influenced by differences in TE identity (see above) and requires additional conditions that seem restrictive, such as haploinsufficiency of siRNA-mediated TE suppression. (We know of no current evidence for or against this condition.) Other expectations about when and where TE misexpression might be most important are more challenging to predict at present. For example, differentiation of TEs is arguably more contingent on population subdivision than are some other potential hybrid incompatibilities; however, more theoretical and empirical

attention will be necessary to address such questions conclusively.

Drawing from specific molecular models, in this review we have focused on mechanisms described in plants. Similar inferences could be tailored to RNA-mediated TE silencing mechanisms in animals. Indeed, both plants and animals appear to have similar small RNA silencing pathways; for example, while PIWI components have not been found in plants, divergent RNA polymerases that are responsible for small RNA amplification could replace PIWI proteins [63]. Still, as noted above, the lack of TE transposition in animal hybrids has created speculation as to whether TEs could play a role in the evolution of animal hybrid incompatibilities. Hybrid inviability associated with TE misregulation has not been observed in *Drosophila*, although the *Drosophila* studies that describe hybrid dysgenesis used lines from the same species that differ in the presence of relatively few TEs [6–8]. The involvement of TEs in *Drosophila* hybrid problems remains, therefore, to be established. We note, however, that the detection ability of TE-mediated hybrid problems might be greater in the endosperm because embryos can be "rescued" from failed endosperm (via independent culture), but *Drosophila* larvae cannot be rescued from failed eggs. In addition, the most detailed studies of hybrid problems in *Drosophila* (in the melanogaster-simulans group) are all crosses involving species with small genomes, and relatively few TEs. (Some *Drosophila* appear to have large genomes due to TEs, but these are not the species used to study speciation.) Still, it is possible that TEs are more relevant to plants; on the whole, plant genomes have many TEs, and even closely related species can differ substantially in their TE complement. As yet, it remains an open question whether TEs will be a "specialist" speciation mechanism or can apply broadly across sexually reproducing organisms.

Finally, we argue that, in order to more convincingly connect TE activity with postzygotic isolating barriers, three pieces of empirical evidence will be essential: lineage differences in TE load and/or identity; clear associations between this differentiation and the expression of hybrid incompatibilities; functional evidence for a mechanistic link between these two phenomena. These data can be obtained through current or rapidly emerging approaches in genetics and genomics. As such, the field is now well positioned to evaluate the connection, if any, between the misregulation of TEs and the expression of hybrid problems—a long-held, intriguing, but poorly assessed mechanism of speciation.

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*Research Article*

# DNA Barcoding and Molecular Phylogeny of *Drosophila lini* and Its Sibling Species

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*Drosophila lini* and its two sibling species, *D. ohnishii* and *D. ogumai*, are hardly distinguishable from one another in morphology. These species are more or less reproductively isolated. The mitochondrial *ND2* and *COI-COII* and the nuclear ITS1-ITS2 regions were sequenced to seek for the possibility of DNA barcoding and to reconstruct the phylogeny of them. The character-based approach for DNA barcoding detected some diagnostic nucleotides only for monophyletic *D. ogumai*, but no informative sites for the other two very closely species, *D. lini* and *D. ohnishii*, of which strains intermingled in the molecular phylogenetic trees. Thus, this study provides another case of limited applicability of DNA barcoding in species delineation, as in other cases of related *Drosophila* species. The molecular phylogenetic tree inferred from the concatenated sequences strongly supported the monophyly of the cluster of the three species, that is, the *lini* clade. We propose some hypotheses of evolutionary events in this clade.

## 1. Introduction

Studies of just diverging populations or species shed light on speciation mechanisms. An important evolutionary process in speciation is the diversification of genes between populations. Most comprehensive information on gene (DNA) evolution associated with speciation has been accumulated for the *Drosophila melanogaster* species subgroup, especially the *D. simulans* clade (e.g., [1–6]), and the *D. obscura* species group [7, 8]. In comparison, speciation mechanisms have been less explored in the *D. montium* species subgroup, in spite of its highest species diversity (= 89) [9] in the *melanogaster* group, with a variety of species at different stages of speciation process. On the other hand, molecular markers have been used to detect cryptic species under incipient speciation process. In the *D. montium* subgroup as well, such a molecular approach has recently been employed to reveal the presence of a cryptic species in the *D. serrata* species complex [10]. "DNA barcoding" is proposed as a promising tool not only for rapid identification of known species, that is, "species identification," but also for discovery and delimitation of species, that is, "species discovery" or "DNA taxonomy" [11–13].

Discovery of a sibling species of *Drosophila lini* is one of cases in which molecular characters were used for "species discovery" in early days. It was first recognized as a species closely related to but different from *D. lini* based on the results of electrophoretic analyses [14]. Then, the studied "*D. lini*-like" strain, MMY326, from Pyinoolwin in central Myanmar, along with another strain (MMY307) from the same locality, was described as *D. ohnishii* [15]. At the same time, another sibling species, *D. ogumai*, was described for two strains (RGN3 and RGN206) from southern Myanmar [15]. It is, however, hard to morphologically distinguish among the three species, *D. lini*, *D. ohnishii,* and *D. ogumai*, especially between the former two, although 80–100% correct classification was achieved for them by discriminant analyses using 13 or 15 quantitative characters [15]. The

evidence from cross-tests supports the presence of three sibling species. More or less strong postmating isolation is present among them: no F1 hybrids could be obtained from crosses between *D. ogumai* and *D. ohnishii*, while the other interspecific crosses produced fertile hybrid females but sterile males [16]. In addition, strong premating isolation was detected between *D. ohnishii* and *D. lini* or between *D. ohnishii* and *D. ogumai*, but not between *D. lini* and *D. ogumai* [16–18]. When the *D. kikkawai* species complex was established in the *D. montium* species subgroup of the *D. melanogaster* species group, *D. lini* was included in it [19]. Subsequent molecular phylogenetic studies consistently supported the close relationships between *D. lini* (and its siblings) and *D. kikkawai* (and its siblings, *D. bocki* and *D. leontia*) [14, 20–24]. However, the relationships between *D. lini* and its siblings have not been resolved yet.

Up to date, it is known that *D. ohnishii* is distributed in central Myanmar to southwestern China (Xishuangbanna), just occupying the intermediate range between the ranges of the two allopatric species, *D. lini* distributed in southern China to Taiwan and *D. ogumai* in southern Myanmar [18]. Thus, the premating isolation is seen between the parapatric neighbors of the three species. Based on this biogeographical evidence, a hypothesis that the premating isolation has evolved through the process of reinforcement in the secondary contact zone between *D. ohnishii* and either neighboring species has been proposed [18]. To test or refine this hypothesis, the present study aims at revealing reliable phylogenetic relationships among these three species based on DNA sequence data. In addition, molecular diagnostics are searched to apply the DNA barcoding "species identification" to these sibling species that are hard to be distinguished morphologically from one another.

When focusing on very closely related species, one should select rapidly evolving regions, for example, mitochondrial genes [25] or nuclear rDNA internal transcribed spacer (ITS) [26], as markers. The mitochondrial cytochrome *c* oxidase subunit I (*COI*) gene has been widely used as DNA barcoding for "species identification": its 648-base pair (bp) fragment is the standard marker in the Barcode of Life project [11, 12]. In the present study, we employed two mitochondrial loci, NADH dehydrogenase subunit 2 (*ND2*) and cytochrome *c* oxidase subunit I and II (*COI-COII*), and one nuclear locus, rDNA internal transcribed spacer 1 and 2 (ITS1-ITS2), to examine the phylogenetic relationships among isofemale strains of *D. lini* and its sibling species and to find possible molecular diagnostics for each species of them.

## 2. Materials and Methods

Seven isofemale strains of *D. lini*, four strains of *D. ohnishii,* and two strains of *D. ogumai* were used as focal OTUs, and one strain each of *D. kikkawai*, *D. bocki*, *D. leontia,* and *D. barbarae* of the *kikkawai* complex, and one strain each of *D. jambulina* and *D. seguyi* belonging to the *montium* subgroup were added as ingroup OTUs (Table 1). Three of these isofemale strains (MLN24 and MLN45 of *D. ohnishii* and MLN260 of *D. barbarae*) were established in 2003 from Menglun, southern part of Yunnan Province, China, but all the others derived from the stocks of Tokyo Metropolitan University and have been maintained in laboratory on cornmeal-malt medium at 23°C under continuous light for more than 12 years. The species status of the closely related species, that is, *D. lini*/*D. ohnishii*/*D. ogumai* and *D. kikkawai*/*D. bocki*/*D. leontia*, was confirmed by cross-tests in previous studies [16, 27].

Table 1: List of experimental strains.

| Species | Lines | Collection locality |
|---|---|---|
| *D. lini* | 3146.1 | Taiwan, China |
| | DHS315 | Dinghushan, Guangdong, China |
| | DHS410 | Dinghushan, Guangdong, China |
| | DHS501 | Dinghushan, Guangdong, China |
| | NKS9212 | Nankunshan, Guangdong, China |
| | NKS9231 | Nankunshan, Guangdong, China |
| | NK9242 | Nankunshan, Guangdong, China |
| *D. ohnishii* | MMY309 | Pyinoolwin, Myanmar |
| | MMY326 | Pyinoolwin, Myanmar |
| | MLN24 | Menglun, Yunnan, China |
| | MLN45 | Menglun, Yunnan, China |
| *D. ogumai* | RGN3 | Yangon, Myanmar |
| | RGN206 | Yangon, Myanmar |
| *D. bocki* | Y163 | ? |
| *D. leontia* | AO-2 | ? |
| *D. kikkawai* | OGS4 | ? |
| *D. barbarae* | MLN260 | Menglun, Yunnan, China |
| *D. jambulina* | NH115 | ? |
| *D. seguyi* | K2 | ? |

Table 2: Target regions and primer sequences in the present study.

| Target region | Primer sequence (5′–3′) | Length (bp) |
|---|---|---|
| Mitochondrial loci | | |
| *ND2* | AAGCTACTGGGTTCATACC | 926 |
| | ATATTTACAGCTTTGAAGG | |
| *COI-COII* | ATACCTCGACG(AT)TATTGA | 842 |
| | GTTTAAGAAACCAGTACTTG | |
| Nuclear locus | | |
| *ITS1-ITS2* | TCCGTAGGTGAACCTGCGG | 650 |
| | GTTAGTTTCTTTTCCTC | |
| Total | | 2418 |

Total DNA was extracted from a single fly using a rapid method [28]. The target regions (Table 2) were amplified on an iCycler Thermal Cycler (Bio-Rad) with the PCR cycle program comprised a 5 min of predenaturation at 94°C, 35 cycles of amplification (1 min of denaturing at 94°C; 1 min of annealing at 53°C for *COI-II* and *ND2*, 56°C for ITS1-ITS2, 1 min of extension at 72°C), and final extension at 72°C for 5 min. The amplicons were purified by precipitation with isopropanol and then subjected to sequencing reaction using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) following the recommended protocol.

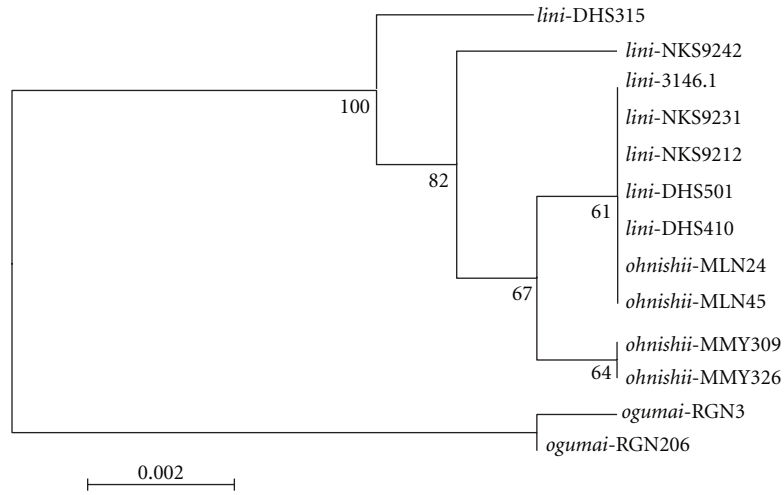


Figure 1: Neighbor-joining (NJ) tree inferred from *ND2* sequences of 13 strains of *D. lini* and its sibling species (*D. ohnishii* and *D. ogumai*). Numbers below branches indicate the bootstrap percentages.

The sequences were analyzed on the 3100-Avant Genetic Analyzer (Applied Biosystems).

The ITS1-ITS2 sequences of three species of the *montium* subgroup were downloaded from GenBank, AY278412 for *D. barbarae*; AY278419 for *D. jambulina,* and AY278431 for *D. seguyi*. For the three species of the *D. melanogaster* subgroup employed as outgroups, the corresponding sequences were also downloaded from GenBank: AF200829 for *ND2* and *COI-COII* and M21017 for ITS1-ITS2 of *D. melanogaster*; AF200846 for *ND2* and *COI-COII* and Z28413 for ITS1-ITS2 of *D. simulans*; AF200831 for *ND2* and *COI-COII*, Z28538 for ITS1-ITS2 of *D. mauritiana*.

DNA sequences were edited and analyzed using MEGA 5.05 [29]. Phylogenetic trees were constructed by the Neighbor-Joining (NJ) method with bootstrap test (1000 replicates) using the Kimura 2-parameter model, with gaps treated by pairwise deletion. For searching DNA barcoding diagnostics, we focused only on the three sibling species with multiple test strains, and applied both of tree- and character-based methods to each of different loci separately. We used the phylogeny-based approach in the former method, examining the monophyly of each species on a phylogenetic tree [30–33]. The character-based method identifies a set of diagnostic nucleotides in the DNA barcode sequence: the four standard nucleotides (A, T, C, G) if found in fixed states in one species can be used as simple pure diagnostics for identifying that species [34]. To examine molecular genealogies for the focal OTUs, we constructed an NJ tree based on the concatenated sequences of the three loci, and applied an estimated divergence time, 5.4 million years ago (Mya) [35], between *D. melanogaster* and *D. simulans* as a calibration point to estimate the divergence time of each node. Before the analysis using the concatenated sequence data, we conducted a Bayesian concordance analysis to test the concordance among the three regions, that is, *ND2*, *COI-COII*, and ITS1-ITS2, using BUCKy [36]. The DNA sequences of each region were analyzed using MrBayes 3.1.2 [37] for Bayesian phylogenetic estimation. Firstly, phylogenetic trees were constructed for each region via the Markov chain Monte Carlo (MCMC) method (number of generations for runs = 1,000,000, nucleotide substitution model = GTR (general time-reversible)), and then, the output of MrBayes was summarized using the mbsum program of BUCKy, and the primary concordance tree was generated with sample-wide concordance factors using default setting in BUCKy.

## 3. Results

*3.1. DNA Barcoding for D. lini and Its Sibling Species.* We sequenced the *ND2* gene in *D. lini* and its siblings and some other species of the *montium* subgroup. The whole sequence of this gene is 1206 bp in most species of the *D. obscura* species group [38]. Our obtained sequences covered most of this region (from the site 34 to 959). The alignment of the sequences included no indel. The GenBank accession numbers of these sequences are AY739939-AY739956. The NJ tree for 13 strains of *D. lini* and its siblings showed that *D. ogumai* was monophyletic but that *D. lini* and *D. ohnishii* were nonmonophyletic with overlap of strains of these two species (Figure 1). There were 20 informative sites in the aligned 13 sequences of *D. lini* and its siblings. Of these sites, 11 nucleotides were specific to *D. ogumai*, and thus can be used as diagnostic nucleotides for identification of this species among the siblings (Table 3). However, there was no species-specific, fixed nucleotide for either *D. lini* or *D. ohnishii*.

The whole *COI* and *COII* sequences are 1536 and 684 bp, respectively, in *D. yakuba* [39]. The *COI-COII* region we sequenced covered 130 bp of *COI* and 639 bp of *COII*. The GenBank accession numbers of these sequences are AY737604-AY737622. The NJ tree based on the *COI-COII* sequences of the 13 strains of *D. lini* and its siblings showed the monophyly of *D. ogumai* but nonmonophyly for either *D. lini* or *D. ohnishii* (Figure 2). Twelve informative sites were detected from this region, among which five were species-specific, diagnostic nucleotides for *D. ogumai* (Table 4). The

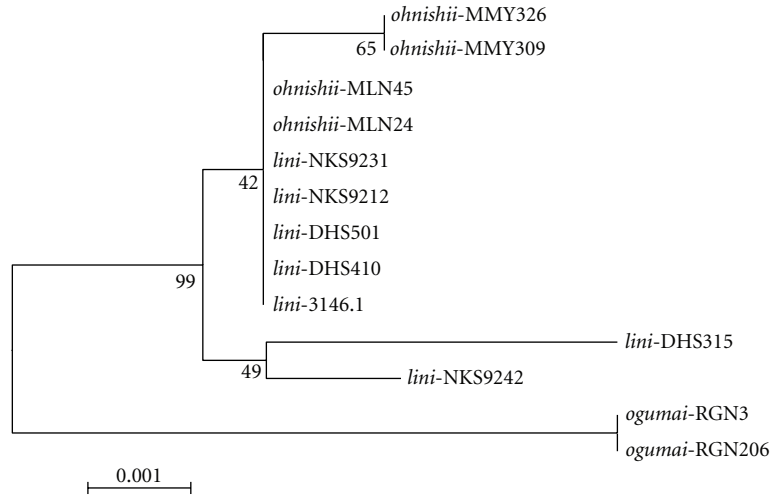


Figure 2: Neighbor-joining (NJ) tree inferred from *COI-COII* sequences of 13 strains of *D. lini* and its sibling species (*D. ohnishii,* and *D. ogumai*). Numbers below branches indicate the bootstrap percentages.

Table 3: Nucleotides at 20 informative sites in *ND2* sequences of 13 strains of *D. lini* and its sibling species (*D. ohnishii* and *D. ogumai*). Diagnostic nucleotides for DNA barcoding are indicated with an asterisk. *N* is the number of strains sequenced. The positions of nucleotide sites are based on the sequence of *D. obscura* [38]. Polymorphic sites are shown with code letters R (A/G,), Y (T/C), S (C/G), W (A/T), and M (A/C).

| Species | *N* | Position in *ND2* (sites 34–959) | | | | | | | | | | | | | | | | | | | | Phylogeny |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 3 | 4 | 4 | 4 | 4 | 5 | 5 | 6 | 6 | 7 | 7 | 7 | 8 | 8 | 9 | 9 | 9 | |
| | | 1 | 5 | 4 | 4 | 0 | 1 | 3 | 8 | 4 | 5 | 6 | 6 | 4 | 8 | 9 | 2 | 9 | 0 | 3 | 5 | |
| | | 4 | 5 | 2 | 5 | 8 | 1 | 4 | 7 | 3 | 2 | 7 | 9 | 7 | 1 | 6 | 9 | 5 | 0 | 4 | 4 | |
| *D. lini* | 7 | R | T | C | A | Y | A | G | S | T | A | W | W | R | T | C | M | C | T | C | C | Non-monophyletic |
| *D. ohnishii* | 4 | A | T | C | A | C | A | G | S | T | R | A | A | G | T | C | A | C | T | C | C | Non-monophyletic |
| *D. ogumai* | 2 | G | C* | T* | R | T | G* | A* | G | C* | A | A | A | G | C* | T* | A | T* | C* | T* | T* | Monophyletic |

Table 4: Nucleotides at 12 informative sites in *COI-COII* sequences of 13 strains of *D. lini* and its sibling species (*D. ohnishii and D. ogumai*). Diagnostic nucleotides for DNA barcoding are indicated with an asterisk. The positions of nucleotide sites are based on the whole length of the *COI* (1536 bp) and *COII* (684 bp) sequences of *D. yakuba* [39]. See Table 3 for further explanations.

| Species | *N* | Position in *COI* (sites 1407–1536) | | | Position in *COII* (sites 1–639) | | | | | | | | | Phylogeny |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1407 | 1485 | 1503 | 69 | 72 | 231 | 232 | 234 | 399 | 435 | 486 | 570 | |
| *D. lini* | 7 | C | Y | T | T | C | C | T | R | Y | W | A | R | Non-monophyletic |
| *D. ohnishii* | 4 | C | C | T | T | C | C | Y | A | T | A | A | A | Non-monophyletic |
| *D. ogumai* | 2 | Y | T | C* | C* | T* | T* | T | A | T | A | G* | A | Monophyletic |

character-based approach failed to distinguish between the two non-monophyletic species for the *COI-COII* sequences as well.

Sequences of the ITS region covering a part of ITS1, the whole 5.8S rDNA, ITS2a, 2S rDNA, and a part of ITS2 were amplified from 10 strains of *D. lini* and its siblings and some other species of the *montium* subgroup. The positions of nucleotides in the sequence were determined by alignment with the ITS sequence of *D. simulans* [26]. The GenBank accession numbers for these sequences are AY739939-AY739956. The 5.8S rDNA, ITS2a, and 2S rDNA were very conservative in all compared species, without variation in the sequence length. On the other hand, the ITS1 and ITS2 diverged largely in respect of either nucleotide substitution or sequence length. The NJ tree for the 10 strains of *D. lini* and its siblings showed the monophyly of *D. ogumai* but non-monophyly for either *D. lini* or *D. ohnishii* (Figure 3). Four informative sites were present in this region,


Figure 3: Neighbor-joining (NJ) tree inferred from ITS1-ITS2 sequences of ten strains of *D. lini* and its sibling species (*D. ohnishii* and *D. ogumai*). Numbers below branches indicate the bootstrap percentages.

Table 5: Nucleotides at four informative sites in ITS1-ITS2 sequences of ten strains of *D. lini* and its sibling species (*D. ohnishii* and *D. ogumai*). Diagnostic nucleotides for DNA barcoding are indicated with an asterisk. The positions of nucleotide sites are based on the sequence of *D. ogumai*. Determination of each region in the sequence is based on the whole sequence of *D. simulans* (Z28413), ITS1: 690 bp; 5.8S: 123 bp; ITS2a: 26 bp; 2S: 30 bp; ITS2: 383 bp [26]. The partial or whole sequence of each region was obtained in *D. ogumai* as: ITS1: last 81 bp; 5.8S: 123 bp; ITS2a: 28 bp; 2S: 30 bp; ITS2: first 392 bp. See Table 3 for further explanations.

| Species | *N* | Position | | | | Pylogeny |
|---|---|---|---|---|---|---|
| | | ITS1 (last 81 bp) | | ITS2 (first 392 bp) | | |
| | | 16 | 307 | 312–325 | 326 | |
| *D. lini* | 6 | G | T | GTCAATAATAAAAT | — | Non-monophyletic |
| *D. ohnishii* | 2 | G | T | GTCAATAATAAAAT/deletion | — | Non-monophyletic |
| *D. ogumai* | 2 | A* | G* | GTCAATAATAAAAT | T* | Monophyletic |

of which two nucleotide substitutions and one insertion were diagnostic for *D. ogumai* (Table 5); the remaining one, a 14-bp indel (sites 312–325) of ITS2, was polymorphic in *D. ohnishii*. For this region as well, no diagnostic nucleotide was found in either *D. lini* or *D. ohnishii*.

*3.2. Molecular Phylogeny.* The primary concordance tree (Figure 4) resulting from the Bayesian concordance analysis for the three loci (*ND2*, *COI-COII*, and ITS1-ITS2) was not discordant, especially the same for the strains of *D. lini* and its sibling species, in topology from the NJ tree (Figure 5) constructed using the concatenated sequences of the three regions (*ND2* + *COI-COII* + ITS1-ITS2, 2442 bp in length), indicating that the mitochondrial and nuclear loci are concordant in the genealogies. The estimated divergence times based on a calibration point of 5.4 Mya divergence between *D. melanogaster* and *D. simulans* [35] were also shown in Figure 5. The three focal sibling species, *D. lini*, *D. ohnishii,* and *D. ogumai*, formed a monophyletic group supported by a high bootstrap value, 96%. This clade (henceforth termed the *lini* clade) formed another, strongly supported (100%) clade with *D. kikkawai* and its siblings, *D. leontia* and *D. bocki*, although the monophyly of the latter three sibling species was not supported. The relationships between the *lini-kikkawai* clade, *D. barbarae* (another species sampled from the *kikkawai* complex), and *D. jambulina* of the *jambulina* complex were not resolved. Within the *lini* clade, two distinct subclades, *D. ogumai* and *D. lini* + *D. ohnishii*, were recognized, with high support values, 100% and 98%, respectively. Within the subclade of *D. lini* + *D. ohnishii*, either species did not form a monophyletic branch: the strain DHS315 of *D. lini* branched off first (bootstrap value 93%), followed by the strain MMY326 of *D. ohnishii* (66%), but there was no nucleotide variation in the concerned sequences among the rest strains including those of *D. lini* from Taiwan, Dinghushan (DHS) and Nankunshan (NKS) in Guangdong Province, and MLN24 of *D. ohnishii* from southern Yunnan.

The ancestor of the *lini* clade was estimated to have appeared about 2.23 Mya. Within the *lini* clade, the divergence between *D. ogumai* and *D. lini* was estimated to have first occurred 1.42 Mya, and then *D. ohnishii* was estimated to have speciated from *D. lini* very recently, at least after 0.17 Mya.

## 4. Discussion

In this study, we tested the applicability of DNA barcoding "species identification" to the *lini* clade consisting of three sibling species, which are morphologically almost indistinguishable [15] but have proved to be more or less reproductively isolated from one another [16, 18]. We took two approaches, the phylogeny-based and character-based methods for DNA barcoding "species identification." However, neither method succeeded in identifying all these three species. The phylogeny-based method revealed the monophyly of *D. ogumai* and the character-based method found some

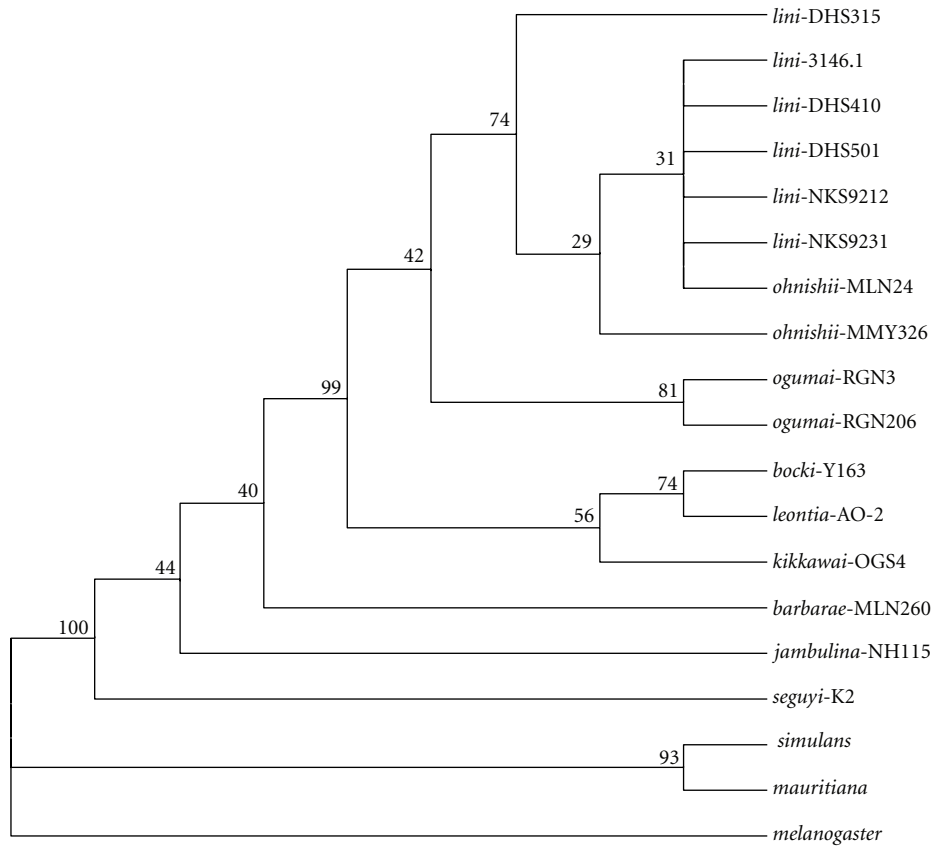


Figure 4: The primary concordance tree resulting from the Bayesian concordance analysis for the three regions, *ND2*, *COI-COII*, and ITS1-ITS2. Numbers above branches are the concordance factors.

diagnostic nucleotides for *D. ogumai*, which can be, if not easily, distinguished from the other two species by a few morphological diagnostic characters [15].

However, we failed to get informative sites for DNA barcoding of two very closely related species, *D. lini* and *D. ohnishii*. This provides another evidential case that DNA barcoding is not always effective in species delineation, which has been corroborated in a number of cases of the genus *Drosophila* as a model system [40]. One possible problem is what genes are to be selected for DNA barcoding. Machado and Hey [41] pointed out that the well-established mutual monophyly of two closed related species, *D. pseudoobscura* and *D. persimilis*, was not recovered by phylogeny reconstruction based on nonrecombining molecules (particularly mitochondrial genome), but was strongly supported by that based on recombining molecules (five X-linked loci). The reason for the former is gene introgression between the species [41]. This may or may not be the case between *D. lini* and *D. ohnishii* as well. On the other hand, recombining molecules (inversions regions) may have contributed to the speciation process by affecting the hybrid fitness [42]. So-called speciation genes involved in the pre- and postmating isolations might be good candidate genes for DNA barcoding and, of course, are very important to understand speciation mechanisms of such species at initial speciation. However, DNA barcoding based on such a standard marker as the 648- bp fragment of *COI* in the Barcode of Life project [11, 12] should be a promising tool for nonexperts to easily and rapidly identify most of known species.

The inferred phylogenetic tree based on the concatenated sequences of the three regions did not support the monophyly of the *kikkawai* complex, although the taxon sampling was quite limited, covering only seven out of 12 species of this complex, in this study. Other studies, though under limited taxon sampling as well, suggested nonmonophyly of this species complex [14, 22, 23, 43]. The delimitation of this species complex should be revised on the basis of molecular phylogenetic analyses under more comprehensive taxon sampling. However, the tree strongly supported the *lini* clade comprising the three sibling species, *D. lini*, *D. ohnishii,* and *D. ogumai*, and placed it close to *D. kikkawai* and its sibling species, in consistence with previous studies [14, 20–23].

With respect to the evolution of the *lini* clade, a hypothesis that the premating isolation has evolved through the process of reinforcement in the secondary contact zone between parapatric neighbors, *D. ohnishii*/*D. lini* or *D. ohnishii*/*D. ogumai*, has been proposed, since the premating isolation is absent between allopatric species, *D. lini* and *D. ogumai* [18]. Based on the phylogeny inferred from the present study and all available biological information from previous studies, we refine or revise the above hypothesis.

The ancestor of the *lini* clade should have derived as a close relative to *D. kikkawai* and/or its sibling species about 2.23 Mya presumably in the subtropics of the Oriental

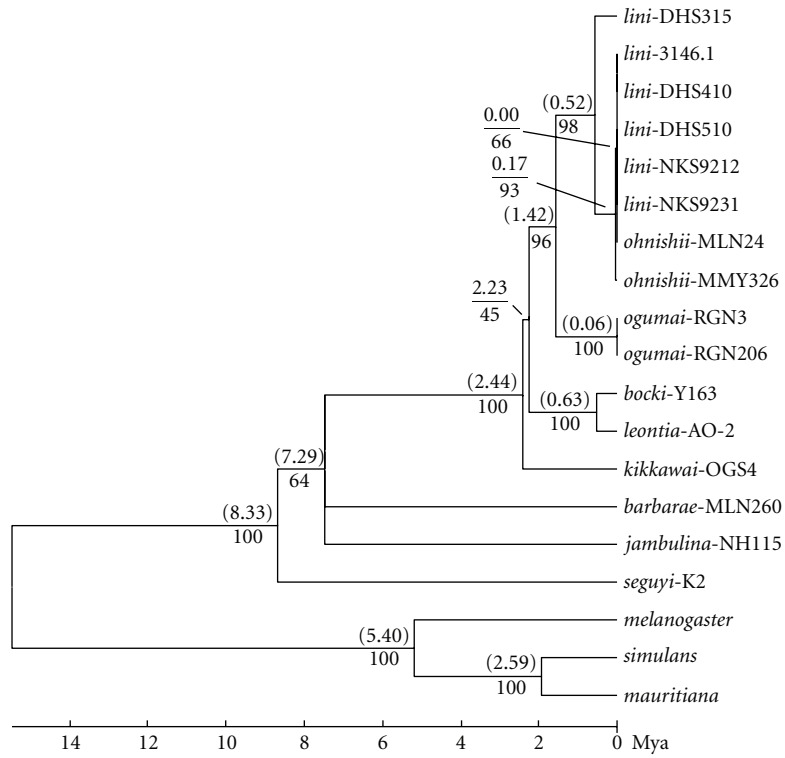


Figure 5: NJ tree inferred from the concatenated sequences (2442 bp) of three regions, *ND2* (926 bp), *COI-COII* (842 bp), and ITS1-ITS2 (674 bp). The time scale (in Mya) was given to the tree on the basis of an estimated time, 5.4 Mya, for the divergence between *D. melanogaster* and *D. simulans* [35] as a calibration point. MEGA 5.05 [29] was used for constructing the tree (bootstrap test: 1000 replications; model: Kimura 2-parameter; gaps: treated by pairwise deletion). Numbers in parentheses above branches indicate divergence times (Mya), and those below branches bootstrap percentages.

Region. Then, the first speciation event producing *D. ogumai* and *D. lini* may have occurred about 1.42 Mya, and, finally, *D. ohnishii* may have diverged from *D. lini* very recently (at least after 0.17 Mya). This speciation order seems to be congruent with the morphological differentiation among the three species: *D. ogumai* can be distinguished from the other two species by a few diagnostic qualitative characters of the male genitalia and also is most remote from the other two species in terms of morphological distance based on metric characters [15]. In addition, variation in the strength of postmating isolation among the three species seems to be congruent as well with the speculated speciation order: the complete postmating isolation (production of no F1 hybrids) is present between the most diverged species, *D. ogumai* and *D. ohnishii*, while the postmating isolation is partial, producing F1 fertile female but sterile male hybrids, between the other pairs of species [16].

As a cue for the premating isolation caused by female repelling behavior, the frequency of sine song generated by males in their copulatory courtships has strongly been suggested from the evidence that it is different among the three species (significantly lower in *D. ohnishii* than in *D. lini* and *D. ogumai*) in accordance with the modes of sexual isolation between them [18]. Furthermore, wing-cut and playback experiments have provided crucial evidence for that the sine song frequency is used as a sexual cue for mate recognition in the *lini* clade [44, 45]. In light of the inferred phylogeny, it is most parsimonious to consider that the lower frequency sine song has evolved in *D. ohnishii*. If so, the first speciation between *D. ogumai* and *D. lini* should have occurred allopatrically, because sympatric or parapatric speciation seems to be inconceivable under the absence of premating isolation. As for the second speciation of *D. ohnishii* from *D. lini*, two hypotheses can be conceived with respect to the evolutionary sequence of post- and premating isolations: (1) if the postmating isolation has first evolved, it should have been established between geographically isolated populations as in the first speciation event. Then, the premating isolation, that is, the lower frequency sine song, may have evolved through the process of reinforcement in the secondary contact zone with either neighboring species in *D. ohnishii*. Even in the light of the phylogeny inferred from the present study, we cannot determine the secondary contact to which species has promoted this evolution. (2) If the premating isolation has evolved first, the change in frequency of sine song has occurred and fixed in *D. ohnishii* as a consequence of adaptation to specific, but unknown, environmental conditions or as a neutral change irrespective of any adaptation and has secondarily come to function, actually or potentially, as a cue for mate recognition by females in this group. If this is the case, *D. ohnishii* would have speciated from a small local population, where

such (a) mutant gene(s) causing differentiation of sexual character(s) are apt to be fixed, within the range of *D. lini*. However, the mtDNA haplotype polymorphism observed in *D. ohnishii* (Figures 1 and 2) seems to be inconsistent with this hypothesis, although it does not rule out the possibility of gene introgression after speciation, especially from southern China (DHS and NKS) populations of *D. lini* to southwestern China (MLN) population of *D. ohnishii*.

For the establishment of postmating isolation, another possibility is infection of microorganisms that cause cytoplasmic incompatibility [46–48]. However, Wolbachia infection has never been detected from any strains of the *lini* clade (M. Watada, personal communication). From another aspect, however, there remain large areas lacking distribution data between the ranges of the three species, especially between Xishuangbanna, southern Yunnan (the eastmost population of *D. ohnishii*) and Dinghushan, central Guangdong (the westmost population of *D. lini*). Filling this gap of data will prompt us to revise the hypothesis about the evolution of these two species seemingly having diverged very recently. In addition, studies of speciation genes relating to the post- and premating isolations, especially those underlying the differentiation of sine song frequency, are needed.

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*Research Article*

# Postzygotic Isolation Evolves before Prezygotic Isolation between Fresh and Saltwater Populations of the Rainwater Killifish, *Lucania parva*

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Divergent natural selection has the potential to drive the evolution of reproductive isolation. The euryhaline killifish *Lucania parva* has stable populations in both fresh water and salt water. *Lucania parva* and its sister species, the freshwater *L. goodei*, are isolated by both prezygotic and postzygotic barriers. To further test whether adaptation to salinity has led to the evolution of these isolating barriers, we tested for incipient reproductive isolation within *L. parva* by crossing freshwater and saltwater populations. We found no evidence for prezygotic isolation, but reduced hybrid survival indicated that postzygotic isolation existed between *L. parva* populations. Therefore, postzygotic isolation evolved before prezygotic isolation in these ecologically divergent populations. Previous work on these species raised eggs with methylene blue, which acts as a fungicide. We found this fungicide distorts the pattern of postzygotic isolation by increasing fresh water survival in *L. parva*, masking species/population differences, and underestimating hybrid inviability.

## 1. Introduction

There is substantial evidence that adaptation to different environments can lead to the evolution of reproductive isolation between populations, a process referred to as ecological speciation [1–6]. Ecological speciation predicts the evolution of both prezygotic and environmentally dependent postzygotic isolation. Prezygotic isolation can evolve as mating signals and preferences adapt to different environments [7–12]. Extrinsic (environmentally dependent) postzygotic isolation may also evolve since hybrids have intermediate phenotypes and are poorly adapted to parental habitats [13–17]. Currently, there is less evidence that genetic incompatibilities between populations (intrinsic postzygotic isolation) can evolve simply as a consequence of adaptation to different habitats [18–20]. Most identified intrinsic isolating barriers have no clear relationship to adaptation and may have arisen subsequent to ecological divergence [21–23]. However, theoretical and empirical work suggests intrinsic isolation can arise through ecological divergence if there are epistatic interactions between alleles conferring environment-specific adaptations [24–26].

When prezygotic, extrinsic, and intrinsic postzygotic reproductive isolating barriers evolve as byproducts of adaptation, the probability that they will lead to speciation depends on their cumulative strength and ability to persist in the face of gene flow when incipient species come into contact [27]. If the cumulative strength of isolating barriers is insufficient, population divergence will be lost via introgression, and speciation will not occur [28]. Therefore, determining how adaptation generates both pre- and postzygotic isolating barriers and how rapidly these barriers evolve is a key focus of speciation research.

Much previous work has focused on timing and order in which reproductive isolating barriers arise, but has given little consideration to their ecological context. In species of *Drosophila* studied in a common laboratory environment, prezygotic isolation evolves faster than postzygotic isolation

[29, 30]. However, this effect seems to be driven by the effect of sympatry and, in allopatric species, pre- and postzygotic isolation evolve at the same rate. Prezygotic isolation also evolves well before postzygotic isolation in birds [31, 32], salamanders [23], and several groups of fish (including centrarchids [33], African Rift Lake cichlids [34], and darters [35, 36]). For instance, postzygotic isolation in fish appears to accumulate slowly with hybrid inviability not becoming complete until species have been separated for 10 to 20 million years [33, 34]. However, in many of these studies, hybrids are raised in a common laboratory environment, which may underestimate hybrid inviability. Differences in population ecology and how these may relate to the strength of isolating barriers are not usually considered. One exception to this is work on stickleback fish which has found that young stickleback species pairs exhibit prezygotic and environmentally based postzygotic isolation, while older pairs show both prezygotic and intrinsic postzygotic isolation [8, 13, 37, 38].

In our study, we ask which reproductive isolating barriers have evolved between ecologically divergent populations within one species of killifish and compare them to barriers that have evolved between two sister species. This allows us to determine the order in which isolating barriers arise as populations adapt to different ecological conditions. The rainwater killifish, *Lucania parva*, is a euryhaline species with permanent populations existing in fresh, brackish, and salt water across the Southeastern United States [39]. *L. parva*'s sister species, the bluefin killifish (*Lucania goodei*), is found almost exclusively in fresh water in Florida [40]. Sympatric populations of *L. parva* and *L. goodei* can be found in several freshwater sites across Florida. Multiple lines of evidence suggest that adaptation to different salinity conditions has occurred between species. *L. goodei* has higher fitness in fresh water relative to *L. parva*, and *L. parva* fares better in brackish and salt water than *L. goodei* [41]. Additionally, *L. goodei* has a decreased rate of hatching success at high salinities while *L. parva* has a lower rate of survival to adulthood in fresh water [41–43]. However, *L. parva* appears to have equal hatching success with *L. goodei* in fresh water. All this previous work on *L. parva* and *L. goodei* has raised eggs with the fungicide methylene blue [44]. While this fungicide improves hatching success, it may do so disproportionally for different salinities, populations, or species. Therefore, in our study, we raised eggs in water with and without methylene blue.

Reproductive isolation between *L. parva* and *L. goodei* involves both prezygotic and postzygotic barriers. Behavioral isolation is quite strong with *L. parva* and *L. goodei* mating pairs taking longer to produce eggs than conspecific pairs and producing fewer eggs [41]. Postzygotic isolation between species is both extrinsic and intrinsic. Backcrosses, F1, and F2 hybrids have reduced survival, particularly at high salinities. In addition, F1 hybrids sons of *L. parva* females and *L. goodei* males have reduced fertility [43].

Some of these isolating barriers between *L. parva* and *L. goodei* may have arisen due simply to adaptation to fresh and salt water. Life in fresh water and salt water pose different osmoregulatory challenges for aquatic animals. In fresh water, fish need to keep excess water out of their bodies, while retaining vital salts. However, marine fish need to extricate salt, but retain water [45]. Throughout the life of the fish, osmoregulation can occur in the gills, guts, kidneys, and skin [46]. Therefore, adaptation to salinity can potentially cause divergence in many genes involved in ion regulation [47, 48], increasing the likelihood of speciation as a direct consequence of adaptation to salinity [5]. To ask how salinity may drive the evolution of isolation barriers in *Lucania*, we measured isolation between *L. parva* populations adapted to different salinity environments.

We collected *L. parva* from a permanent fresh water population (Pecos River) and a salt water population (Indian River Lagoon). We crossed Pecos and Indian River fish and predicted that if prezygotic isolation existed, between populations, mating pairs would take longer to mate and produce fewer eggs than within population pairs. We

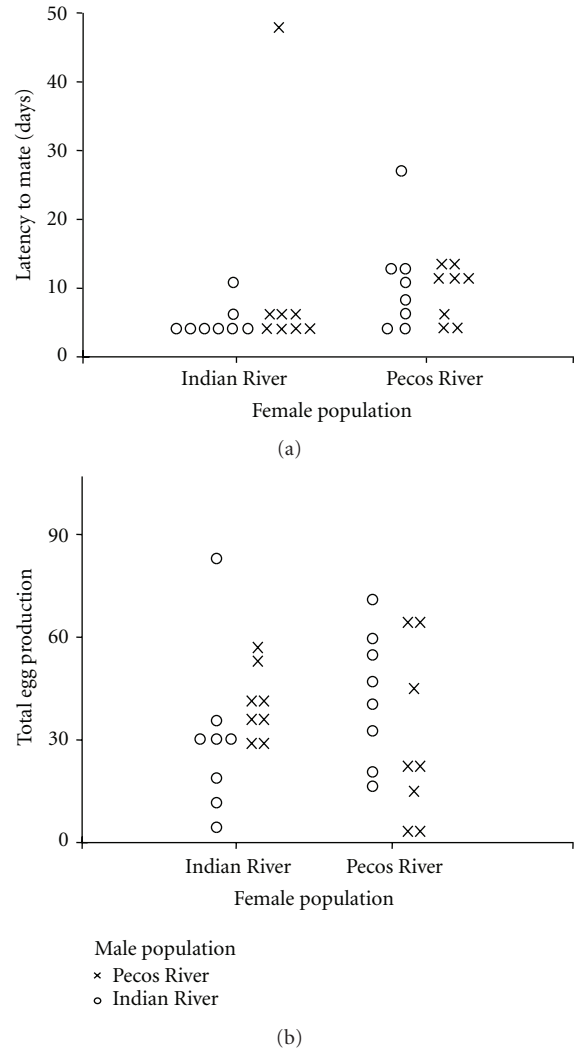


Figure 1: *L. parva* mate preference. Measures of behavioral isolation between populations plotted by female source population. Circles indicate females mated to Indian River males, crosses indicate females mated to Pecos River males. (a) Latency to mate in days (including outlier), (b) total egg production over 61 days.

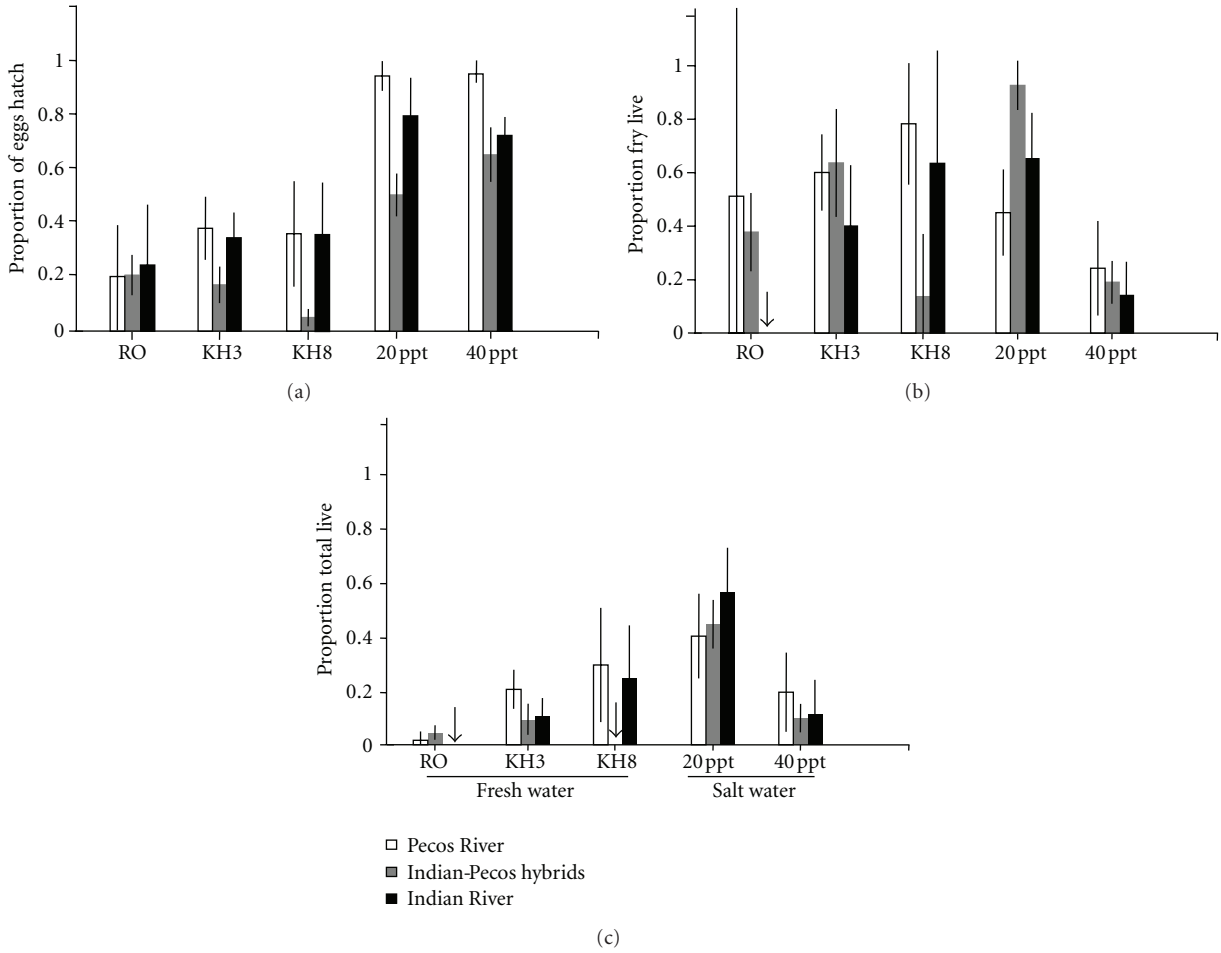


Figure 2: *L. parva* survival differences in fresh and salt water. Mean survival probabilities (± standard error) for Pecos River (white bars), Indian River (black bars), and Indian-Pecos hybrid crosses (gray bars) across different water chemistries: reverse osmosis water (RO), soft water (KH3), hard water (KH8), saline (20 ppt), and hypersaline (40 ppt). All crosses were raised in the absence of methylene blue. Arrows indicate mean survival probability of zero. (a) The proportion of eggs hatched, (b) proportion of fry that survived to 14 days after hatch, (c) total survival (proportion of eggs that survived to 14 days after hatch).

then raised Pecos-Indian River hybrid eggs in five water chemistries ranging from fresh to salt water and measured survival. If postzygotic isolation exists, hybrid eggs and fry should have lower survival than either of the parental populations. Hybrid inviability across environments would be evidence for intrinsic isolation, while environmentally dependent inviability would suggest that isolation is extrinsic. Furthermore, if any local adaptation is present, we would predict the freshwater population to have higher survival than the saltwater population in fresh water treatments and the saltwater population to have higher survival in salt water conditions. We measured survival of our *L. parva* populations with and without methylene blue to determine if the fungicide had any effect on measures of postzygotic isolation.

Additionally, we wished to compare the survival of freshwater, saltwater, and hybrid *L. parva* to *L. goodei* survival. Previous work has established that *L. goodei* has extremely low survival in salt water, but equal survival with *L. parva* in fresh water. However, in these studies, eggs were raised in methylene blue, and only a single fresh water treatment was used [39]. Therefore, we collected eggs from one population of *L. goodei* and raised them in two fresh water treatments in the absence of methylene blue. We predicted that *L. goodei* should have higher survival in fresh water than *L. parva*.

## 2. Methods

We collected *L. parva* from two ecologically different and geographically distant sites: an inland river in Texas and the Atlantic Ocean off the coast of Florida. Our freshwater site was Pecos River, along the Pecos-Crockett County border, TX. At the time of collection, the carbonate hardness (KH, a measure of mineral content) of the water was low (between 3 and 4). However, the upper Pecos River does have a history of salinization due to input from salt springs and dam construction altering water flow, which may contribute to *L. parva*'s persistence there [49]. Our saltwater site was Indian River Lagoon, Brevard County, FL, on the Atlantic

Table 1: Analyses of prezygotic isolation between *L. parva* populations. Results of general linear model for (a) latency to mate, (b) total number of eggs produced. Male population (Pecos, Indian River), female population (Pecos, Indian River), and their interaction are included as factors. Prezygotic isolation predicts an interaction between male population and female population. Statistically significant values are indicated in bold.

(a) Latency to mate

| | Outlier included | | | Outlier removed | | |
|---|---|---|---|---|---|---|
| Source | *df* | *F* | *P* | *df* | *F* | *P* |
| Male population | 1,28 | 0.32 | 0.58 | 1,27 | 0.34 | 0.56 |
| Female population | 1,28 | 0.52 | 0.48 | **1,27** | **9.31** | **0.0051** |
| Male ∗ Female population | 1,28 | 1.17 | 0.29 | 1,27 | 0.18 | 0.68 |

(b) Total number of eggs produced

| Source | *df* | *F* | *P* |
|---|---|---|---|
| Male population | 1,28 | 0.08 | 0.78 |
| Female population | 1,28 | <0.01 | 0.96 |
| Male ∗ Female population | 1,28 | 2.57 | 0.12 |

Table 2: Analyses of *L. parva* survival based on cross type (Pecos, IR, Hybrid) and water chemistry. Results of generalized linear model for (a) proportion of eggs hatched, (b) proportion of fry that survived to 14 days after hatch, (c) total survival (proportion of eggs that survived to 14 days after hatch) in five different water chemistries: reverse osmosis water (RO), soft water (KH3), hard water (KH8), saline (20 ppt), and hypersaline (40 ppt). Statistically significant values are indicated in bold. Number of families per water treatment: RO = 22, KH3 = 25, KH8 = 25, 20 ppt = 25, 40 ppt = 24; total = 121. Number of eggs per water treatment: RO = 220, KH3 = 241, KH8 = 224, 20 ppt = 326, 40 ppt = 241. Number of fry per water treatment: RO = 39, KH3 = 66, KH8 = 44, 20 ppt = 242, 40 ppt = 181.

(a) Proportion of eggs hatched

| Source | *df* | $\chi^2$ | *P* |
|---|---|---|---|
| *Cross* | **2** | **18.83** | **<0.0001** |
| *Water Chemistry* | **4** | **106.48** | **<0.0001** |
| Cross ∗ Water Chemistry | 8 | 8.23 | 0.4116 |

(b) Proportion of fry survive

| Source | *df* | $\chi^2$ | *P* |
|---|---|---|---|
| Cross | 2 | 0.64 | 0.7257 |
| *Water Chemistry* | **4** | **59.73** | **<0.0001** |
| *Cross ∗ Water Chemistry* | **8** | **24.77** | **0.0017** |

(c) Total survival

| Source | *df* | $\chi^2$ | *P* |
|---|---|---|---|
| Cross | 2 | 1.83 | 0.4013 |
| *Water Chemistry* | **4** | **77.47** | **<0.0001** |
| *Cross ∗ Water Chemistry* | **8** | **19.90** | **0.0107** |

coast. Salinity in Indian River is typically 35 ppt. We collected *L. goodei* from the Wakulla River, Wakulla County, FL. At each site, we collected animals using dipnets and seines. The collected fish were transported back to University of Illinois and housed in 75–109 L stock tanks. Indian River (IR) fish were kept in reverse osmosis water raised to 35 ppt salinity using Instant Ocean Sea Salt (Spectrum Brands, Atlanta, GA). They were then transitioned to 10 ppt water, then at the beginning of the experiment to tanks containing city water treated with the dechlorinating agent Start Right (Jungle Laboratories, Cibolo, TX) at 2 ppt salinity. Pecos River fish were kept in treated city water at 2 ppt salinity and with Alkaline Regulator (Seachem, Madison, GA) added to bring the carbonate hardness (KH) to 10. Fish were fed daily *ad libitum* with a mixture of frozen brine shrimp and flake food. Fish were maintained under a light cycle of 14 hours light, 10 hours dark.

We performed both within and between population crosses. We set up four different cross types: two within population crosses (Pecos female by Pecos male, IR female by IR male) and two between population crosses (Pecos female by IR male, IR female by Pecos male). There were 8 replicates of each cross type, for a total of 32 pairings. For each pair, we placed one male and one female in a 38 L tank filled with dechlorinated city water at 2 ppt salinity. Visual barriers were placed between all tanks to isolate mating pairs from others. Four yarn mops were provided as a spawning substrate (two floating and two sinking mops).

The mops were checked for eggs every 2-3 days. All collected eggs were checked under a microscope to verify that they were recently fertilized. Killifish eggs take approximately 7–9 days to hatch; therefore, most eggs (at 1-2 days old) were very early in development when they were transferred to their water treatments. We recorded the number of eggs found on each egg check. Latency to mate was measured over the first 47 days and was calculated as the number of days until the first egg was found. If a pair had not mated after 47 days, we assigned them a latency of 48 days (the total number of days plus 1 day) [41]. After 47 days, we removed visual barriers between tanks to encourage spawning and continued collecting eggs. We summed the total number of eggs laid over the entire experiment (61 days).

Eggs were transferred to small plastic tubs with different water treatments. There were three fresh water treatments: pure reverse osmosis water (RO), soft water (KH3), and hard water (KH8). The RO water was created using a filtration system that removes sediment, chlorine, and other large ions from city water (AquaFx Barracuda 4 Stage RO/DI System, Winter Park, FL). Soft water was created by adding Alkaline Regulator (Seachem, Madison, GA) and R/O Right (Kent Marine, Franklin, WI) to adjust the ionic content of RO water to a carbonate hardness of KH3. Hard water was created by adding Alkaline Regulator and R/O Right to dechlorinated city water until its hardness was KH8. The salt water treatments were made by adding Instant Ocean Sea Salt to RO water until the desired salinity was reached. Ocean water is typically 32 ppt, and we used two salinity treatments: saline (20 ppt) and hypersaline (40 ppt). Additionally, we raised some eggs in the KH8 and 20 ppt treatments with

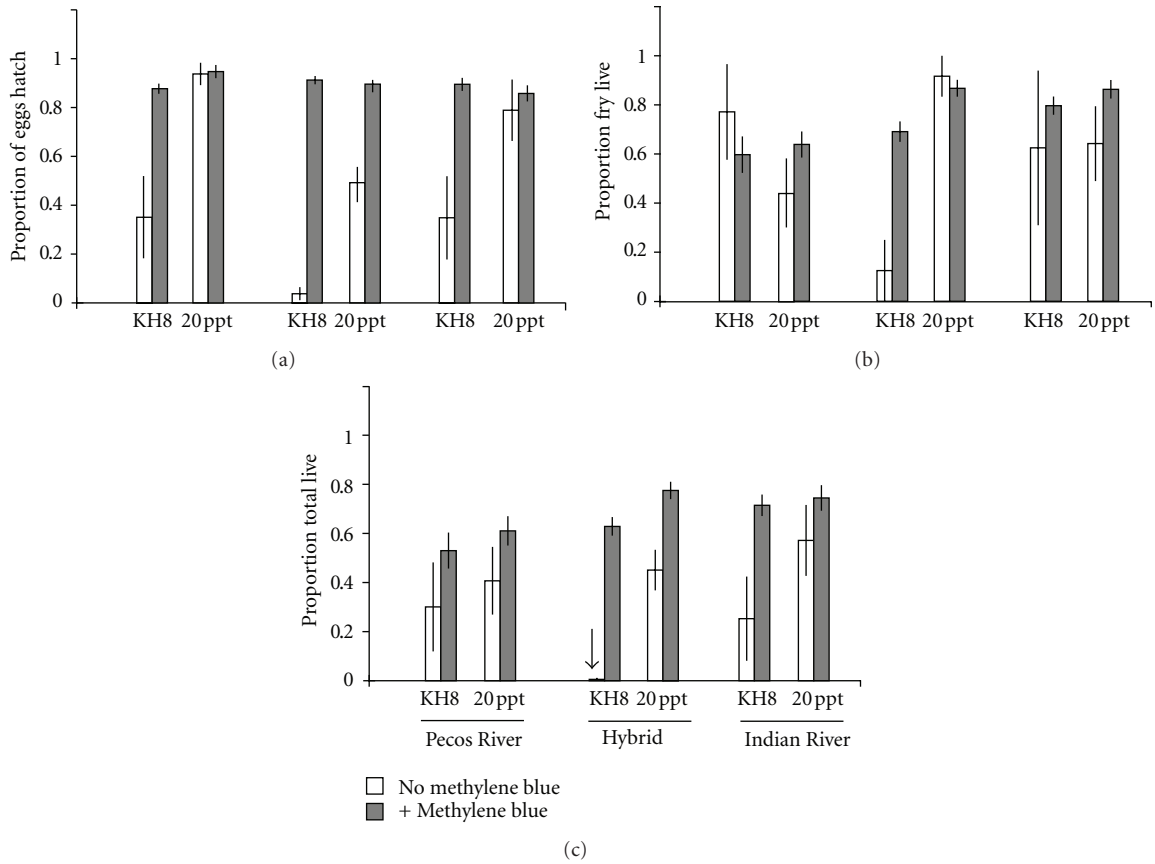


Figure 3: *L. parva* survival differences with methylene blue addition. Mean survival probabilities (± standard error) for Pecos River, Indian River, and Indian-Pecos hybrid crosses in fresh water (KH8) and salt water (20 ppt) with methylene blue addition (gray bars) and without (white bars). Arrows indicate mean survival probability of zero. (a) The proportion of eggs hatched, (b) proportion of fry that survived to 14 days after hatch, (c) total survival (proportion of eggs that survived to 14 days after hatch).

methylene blue, the antifungal agent. A 3 ppm solution of methylene blue ($C_{16}H_{18}N_3SCl$; Kordon LLC, Hayward, CA) was added to the water immediately after eggs were placed in it. We rotated the water treatment every egg collection day to assure an equal distribution of eggs in each water treatment. We collected eggs until each water treatment had at least 10 eggs from each tank. Once the eggs hatched, we transferred the fry into clean tubs with the same water treatments.

Eggs and fry were censused every 2-3 days. We recorded the number of eggs that were alive or dead, the number of eggs hatched, and the number of fry that were alive or dead. These censuses continued until 14 days after hatching, at which point fry were euthanized with an overdose of MS-222. We measured survival in several ways. We measured the proportion of eggs that hatched (hatching success), the proportion of fry that survived to 14 days of age (fry survival), and the proportion of eggs that produced surviving fry of 14 days of age (total survival). These proportions were calculated separately for each family in each water chemistry. We combined the data for the two between population cross types into one hybrid group. We had eggs from 32 families total (Pecos = 8, IR = 8, Hybrid = 16). Not all families had eggs in all water chemistries; therefore, we list sample sizes for each water chemistry in our table legends.

To measure *L. goodei* survival in fresh water in the absence of methylene blue, we collected eggs from *L. goodei* stock tanks (not from the preestablished crosses). Mops from these tanks were checked three times a week. The eggs collected were placed into KH3 or KH8 treatments, both without methylene blue. These eggs were also checked under a microscope to verify they were fertilized. Hatching success and fry survival were measured as described above.

All statistical analyses were performed using SAS statistical software (SAS V 9.1, Cary, NC). Measures of prezygotic isolation (latency to mate and total number of eggs produced) were analyzed in a general linear model with male source population (Pecos, Indian River), female source population (Pecos, Indian River), and the interaction between male population and female population. If behavioral isolation existed, we would expect a significant interaction between male and female population. There was an outlier in our latency to mate data, with one Indian River female by Pecos male taking more than 47 days to mate, so we performed the analysis with and without this outlier to determine if it affected our conclusions.

For survival data, we analyzed the proportion surviving at each life stage for each cross type using generalized linear models assuming a binomial distribution (proc genmod

in SAS) and used maximum likelihood to evaluate the significance of effects. We used the "dscale" option in SAS to control for overdispersion when this occurred [43]. To determine survival in the absence of methylene blue, we used a model that considered the effects of water chemistry (RO, KH3, KH8, 20 ppt, 40 ppt), cross type (Pecos, IR, Hybrid), and their interaction on the probability of hatching, fry survival, and total survival. We also ran analyses where we included family (nested within cross type) as a repeated factor in our general linear model, but it did not alter our results, and these analyses are not presented here.

To determine the effects of methylene blue on survival in hard and 20 ppt, we ran a second model which examined the effects of cross type, water chemistry (KH8, 20 ppt), presence/absence of methylene blue and their interactions on the probability of hatching, fry survival, and total survival.

To compare *L. parva* to *L. goodei* fresh water survival in the absence of methylene blue, we analyzed probability of survival at each stage (egg, fry, total) in fresh water chemistries (KH3, KH8), based on cross type (Pecos, IR, Hybrid, *L. goodei*) and included the interaction between cross type and water chemistry. Means and standard errors are reported throughout for all analyses.

## 3. Results

We found no evidence for prezygotic isolation between Pecos and Indian River fish. Between populations pairs (Pecos male by Indian River female; Indian River male by Pecos female) did not differ from within population pairs in latency to mate or total number of eggs produced (Table 1; Figure 1). Removal of the latency outlier did not alter our conclusions about prezygotic isolation. However, when the outlier was removed, we found there was a difference in latency to mate between female populations with Indian River females mating sooner than Pecos females (IR = 5.00 $\pm$ 1.89 days, Pecos = 9.94 $\pm$ 5.82 days).

Despite a lack of prezygotic isolation, we found that offspring from Pecos-Indian River hybrid crosses had reduced survival. Hybrid eggs had lower hatching success than within population eggs across different water treatments (Table 2(a), Figure 2(a)). The proportion of fry that lived and total survival were also lower for hybrid crosses, but only in hard water (Tables 2(b) and 2(c); Figures 2(b) and 2(c); significant cross by water treatment interaction). However, no reduction in hybrid hatching rates was detected when methylene blue was added to the water treatments (Table 3, Figure 3; significant methylene blue by cross interaction). When methylene blue was present, hybrid offspring survived quite well. There was little evidence for local adaptation in egg and fry survival as we did not detect consistent differences between Pecos and Indian River survival. In both populations, hatching success was higher in salt water than in fresh water.

*L. goodei* eggs hatched more than *L. parva* eggs in fresh water treatments in the absence of methylene blue (Table 4; Figure 4). Total survival of *L. goodei* eggs and fry was also higher than *L. parva* survival in fresh water. These differences seem primarily driven by high survival of *L. goodei* eggs and fry in soft water treatments. These results are in contrast to previous work that found no difference between the species when methylene blue was used.

Table 3: Analyses of *L. parva* survival in the presence or absence of methylene blue (MB) for hard water (KH8) and salt water (20 ppt). Results of generalized linear model for (a) proportion of eggs hatched, (b) proportion of fry that survived to 14 days after hatch, (c) total survival (proportion of eggs that survived to 14 days after hatch). Statistically significant values are indicated in bold. Number of families per water treatment: without MB KH8 = 25, 20 ppt = 25, with MB KH8 = 32, 20 ppt = 32; total = 114. Number of eggs per water treatment: without MB KH8 = 224, 20 ppt = 326, with MB KH8 = 1255, 20 ppt = 1019. Number of fry per water treatment: without MB KH8 = 44, 20 ppt = 242, with MB KH8 = 1129, 20 ppt = 908.

(a) Proportion of eggs hatched

| Source | *df* | $\chi^2$ | *P* |
|---|---|---|---|
| *MB* | **1** | **51.91** | **<0.0001** |
| *Water Chemistry* | **1** | **35.45** | **<0.0001** |
| *MB ∗ Water Chemistry* | **1** | **29.80** | **<0.0001** |
| *Cross* | **2** | **4.27** | **0.014** |
| *MB ∗ Cross* | **2** | **5.39** | **0.0045** |
| Water Chemistry ∗ Cross | 2 | 2.02 | 0.1325 |
| MB ∗ Water Chemistry ∗ Cross | 2 | 0.13 | 0.8821 |

(b) Proportion of fry survive

| Source | *df* | $\chi^2$ | *P* |
|---|---|---|---|
| MB | 1 | 0 | 0.9574 |
| Water Chemistry | 1 | 1.41 | 0.2357 |
| MB ∗ Water Chemistry | 1 | 0.12 | 0.7278 |
| Cross | 2 | 0.96 | 0.3828 |
| MB ∗ Cross | 2 | 0.07 | 0.9285 |
| *Water Chemistry ∗ Cross* | **2** | **7.06** | **0.0009** |
| *MB ∗ Water Chemistry ∗ Cross* | **2** | **4.58** | **0.0103** |

(c) Total survival

| Source | *df* | $\chi^2$ | *P* |
|---|---|---|---|
| *MB* | **1** | **56.55** | **<0.0001** |
| *Water Chemistry* | **1** | **22.27** | **<0.0001** |
| *MB ∗ Water Chemistry* | **1** | **12.04** | **0.0005** |
| Cross | 2 | 2.74 | 0.0644 |
| MB ∗ Cross | 2 | 2.85 | 0.0578 |
| *Water Chemistry ∗ Cross* | **2** | **4.31** | **0.0135** |
| MB ∗ Water Chemistry ∗ Cross | 2 | 2.67 | 0.0692 |

## 4. Discussion

Here, we show that postzygotic isolation has begun to evolve between freshwater and saltwater populations of *L. parva*. However, there is no evidence that any prezygotic isolation yet exists. This suggests that genes involved in hatching success and fry survival evolve more rapidly between

Table 4: Analyses of *L. goodei* and *L. parva* survival in two fresh water chemistries (KH3 and KH8). Results of generalized linear model for (a) proportion of eggs hatched, (b) proportion of fry that survived to 14 days after hatch, (c) total survival (proportion of eggs that survived to 14 days after hatch). Statistically significant values are indicated in bold. Number of families per water treatment: IR KH3 = 6, KH8 = 6; Hybrid KH3 = 13, KH8 = 14; Pecos KH3 = 6, KH8 = 5; *L. goodei* KH3 = 5, KH8 = 7; total = 62. Number of eggs per water treatment: IR KH3 = 79, KH8 = 78; Hybrid KH3 = 94, KH8 = 86; Pecos KH3 = 68, KH8 = 60; *L. goodei* KH3 = 50, KH8 = 31. Number of fry per water treatment: IR KH3 = 21, KH8 = 21; Hybrid KH3 = 14, KH8 = 7; Pecos KH3 = 31, KH8 = 16; *L. goodei* KH3 = 45, KH8 = 13.

(a) Proportion of eggs hatched

| Source | *df* | $\chi^2$ | *P* |
|---|---|---|---|
| *Cross* | **3** | **27.94** | **<0.0001** |
| *Water Chemistry* | **1** | **7.26** | **0.0070** |
| Cross ∗ Water Chemistry | 3 | 5.38 | 0.1452 |

(b) Proportion of fry survive

| Source | *df* | $\chi^2$ | *P* |
|---|---|---|---|
| Cross | 3 | 4.02 | 0.2592 |
| Water Chemistry | 1 | 0.62 | 0.4302 |
| Cross ∗ Water Chemistry | 3 | 6.47 | 0.0908 |

(c) Total survival

| Source | *df* | $\chi^2$ | *P* |
|---|---|---|---|
| *Cross* | **3** | **9.48** | **<0.0001** |
| Water Chemistry | 1 | 1.53 | 0.2162 |
| *Cross ∗ Water Chemistry* | **3** | **3.25** | **0.0209** |

*L. parva* populations than genes involved in mating traits and preferences. Most previous work suggests that pre- and postzygotic isolation evolve at similar rates in allopatric populations [29, 30], but this does not appear to be true in *L. parva*.

When we examined population differences within *L. parva*, we found that F1 hybrids between freshwater and saltwater populations had reduced survival compared to offspring from within population crosses. These effects were most apparent in challenging water chemistries: in fresh water and in the absence of methylene blue. The most drastic reduction of hybrid survival was in hard water (KH8). The lethality of hard water may be due to fungus that grew readily in this water treatment. Fungal infections are a major source of egg mortality and both high salinity and methylene blue can prevent infection, although methylene blue is more effective [44, 50]. Methylene blue may also add ions to the water, which may decrease osmoregulatory stress and may be why methylene blue also increased fry survival at low salinities. This suggests that hybrid eggs were less viable than eggs from within population crosses and physiologically challenging water chemistries revealed this decreased viability. We also showed that *L. parva* has lower survival compared to *L. goodei* in fresh water in the absence of methylene blue. This contradicts previously published results that used methylene blue and found no difference in fresh water survival between species [41–43]. This suggests that F1 hybrids between *L. goodei* and *L. parva* may also have low survival in fresh water, but these effects have been masked by the use of methylene blue in previous studies and hybrid fitness may have been previously overestimated.

The decreased viability of Pecos and Indian River hybrid offspring suggests that intrinsic postzygotic isolation exists between populations. The main difference between Pecos and Indian River populations is their native salinity, suggesting that genetic incompatibilities have arisen as a byproduct of adaptation to saline environments. However, in allopatric populations, any mechanism which causes unique alleles to become fixed has the potential to cause incompatibilities as novel alleles come into contact and interact in hybrids (Dobzhansky-Muller incompatibilities: [18, 51–53]). Intrinsic postzygotic isolation between populations can also arise due to genetic drift [20, 52, 54] or genomic conflict [55]. Pecos and Indian River are geographically distant, separated by more than 2400 km and the Gulf of Mexico. Work on other related species from the *Fundulus* clade has found substantial divergence between east and west Gulf populations, possibly due to genetic drift [56]. Therefore, we are currently working on determining the degree of genetic divergence and phylogenetic relationship between these populations using sequence data. In addition, we are conducting crosses between other ecologically divergent populations from the same geographical region as well as geographically distant but ecologically similar populations. This ongoing work will determine if hybrid inviablity in *L. parva* evolves primarily due to salinity adaptation rather than due to drift.

How isolation arises during the initial stages of speciation, when a single population splits into two and populations begin to diverge, still represents a "missing link" in speciation research [57]. By showing that intrinsic postzygotic isolation has begun to evolve between divergent *L. parva* populations, our work suggests it may have been the first barrier to arise between *L. parva* and its sister species *L. goodei*. Adaption to salinity is primarily physiological and, therefore, may be particularly likely to cause intrinsic isolation through epistatic interactions. Similarly, physiological changes associated with toxic environments also appear to lead to substantial genetic changes between populations and, in some cases, to hybrid inviability [58–61]. Therefore, physiological adaptation may be a primary force leading to postzygotic incompatibilities.

Currently, there are competing ideas about how isolating barriers evolve during speciation. In one proposed scenario, strong prezygotic isolation evolves before strong postzygotic isolation. Thus, prezygotic isolation plays a primary role in preventing interbreeding, and postzygotic isolation slowly completes the process of speciation as decreased hybrid fitness and irreversible genetic incompatibilities accumulate [28, 62, 63]. However, this conclusion is based on studies of species pairs that have already undergone speciation [29, 34, 35], populations that occur in sympatry where reinforcement may have strengthened prezygotic isolation [30, 64, 65], or populations in which feeding and mating

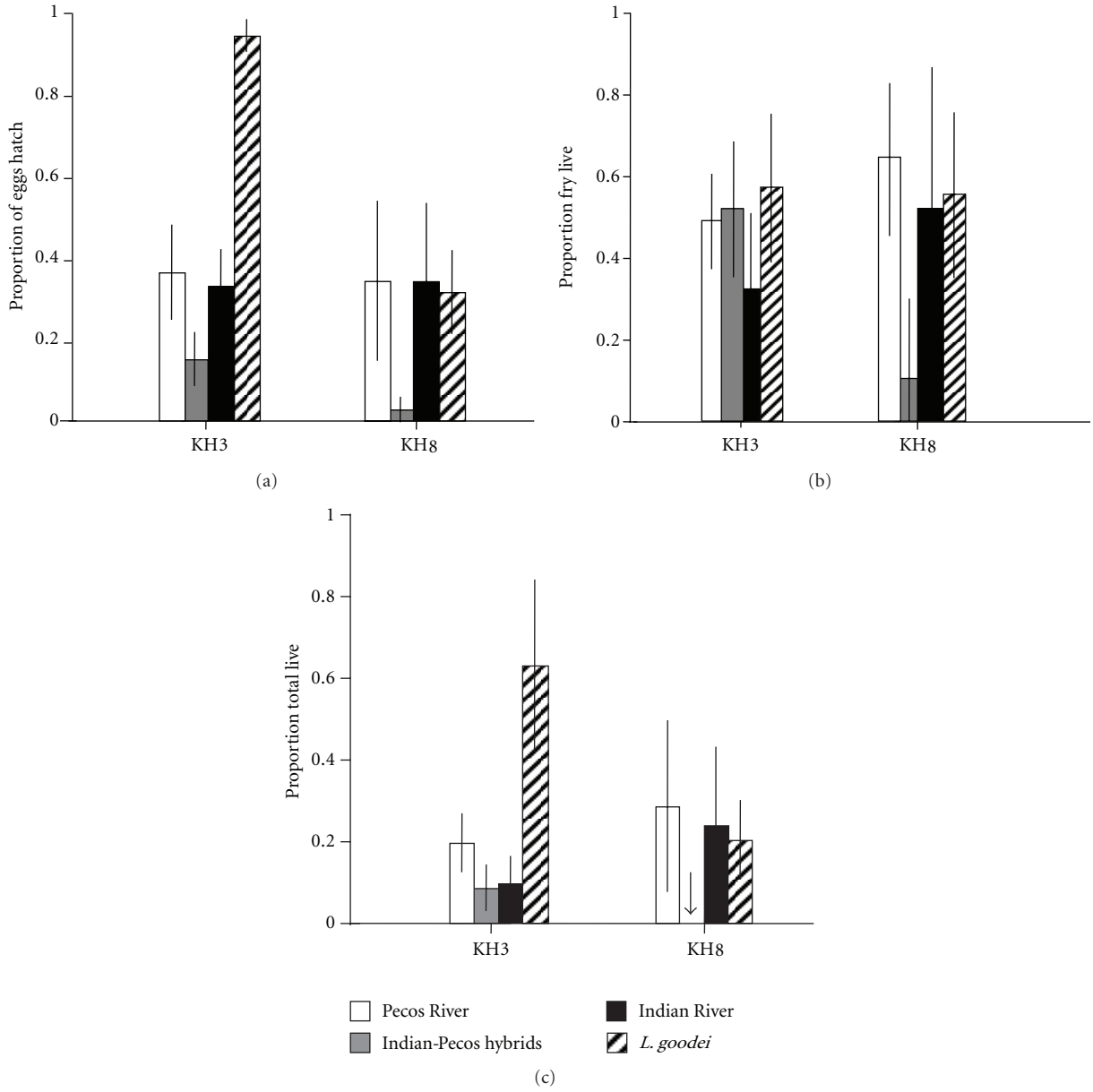


Figure 4: *L. parva* and *L. goodei* survival differences in fresh water. Mean survival probabilities (± standard error) for Pecos River (white bars), Indian River (black bars), Indian-Pecos hybrids (gray bars), and *L. goodei* (hatched bars) crosses in soft (KH3) and hard fresh water (KH8). All crosses were raised in the absence of methylene blue. Arrows indicate mean survival probability of zero. (a) The proportion of eggs hatched, (b) proportion of fry that survived to 14 days after hatch, (c) total survival (proportion of eggs that survived to 14 days after hatch).

occur in the same habitat (such as phytophagous insects [66–68]). Nevertheless, some incipient species do show prezygotic isolation without any postzygotic barriers [57, 69, 70].

There is another possible route to speciation. Genetic divergence might produce hybrid inviability between populations and prezygotic isolation evolves subsequently as divergence continues or as incipient species come into sympatry and reinforcement occurs [18]. When natural selection drives genetic divergence between populations, evolving postzygotic isolation should be primarily environmentally dependent. Many examples of adaptation to divergent environments producing extrinsic isolation exist [1, 4, 15, 17, 19, 71–73], while there are few examples for intrinsic isolation. In a survey of 20 ecologically divergent species pairs, all species exhibited some prezygotic isolation and extrinsic postzygotic isolation, but only three pairs had any documented intrinsic postzygotic isolation [57]. Intrinsic isolation as a result of ecological divergence has only been substantially documented in dwarf and normal lake whitefish [74, 75], copper tolerant plants [58], and an experimental evolution study in yeast [26]. However, few studies distinguish between extrinsic inviability and intrinsic

inviability that appears under stressful conditions [62], such as the decreased viability that appeared in challenging water chemistries in our study. Therefore, future work needs to establish the contribution to divergence of both extrinsic and intrinsic postzygotic isolation and the underlying genetic basis of both. Such work will allow us to determine how postzygotic isolation evolves as a consequence of adaptation, the relative importance of extrinsic and intrinsic barriers, and how postzygotic isolation may act alone or in concert with prezygotic isolation to cause ecological speciation.

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*Research Article*

# Divergent Evolution of Male Aggressive Behaviour: Another Reproductive Isolation Barrier in Extremophile Poeciliid Fishes?

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Reproductive isolation among locally adapted populations may arise when immigrants from foreign habitats are selected against via natural or (inter-)sexual selection (female mate choice). We asked whether also intrasexual selection through male-male competition could promote reproductive isolation among populations of poeciliid fishes that are locally adapted to extreme environmental conditions [i.e., darkness in caves and/or toxic hydrogen sulphide ($H_2S$)]. We found strongly reduced aggressiveness in extremophile *Poecilia mexicana*, and darkness was the best predictor for the evolutionary reduction of aggressiveness, especially when combined with presence of $H_2S$. We demonstrate that reduced aggression directly translates into migrant males being inferior when paired with males from nonsulphidic surface habitats. By contrast, the phylogenetically old sulphur-endemic *P. sulphuraria* from another sulphide spring area showed no overall reduced aggressiveness, possibly indicating evolved mechanisms to better cope with $H_2S$.

## 1. Introduction

*1.1. Ecological Speciation.* Divergent natural selection has the potential to drive adaptive trait divergence along environmental gradients [1], but can also lead to the evolution of reproductive isolating barriers [2, 3]. During ecological speciation, reproductive isolation results from ecologically based divergent selection, and prezygotic isolation may arise as a byproduct of local adaptation if immigrants from ecologically divergent habitats are selected against [3]. This can be owing to natural selection, if immigrants show reduced viability [4–6], or sexual selection, if poorly adapted individuals have a disadvantage in mate competition [5, 7, 8]. Furthermore, ecological speciation may also be driven by selection against hybrids with intermediate phenotypes [9], behavioural isolation based on a "magic trait" [10–14], and sensory drive [15].

Our present paper briefly collates our current knowledge regarding trait divergence and especially mechanisms of reproductive isolation among different locally adapted populations of livebearing fishes (Poeciliidae), currently undergoing ecological speciation processes in response to "extreme" conditions (see below). Using both lab-reared as well as wild-caught fish we then demonstrate that divergent evolution of male competitive abilities (aggressive behaviour) in extremophile fishes may play yet another role in maintaining reproductive isolation among different locally adapted populations: adaptation to extreme habitat conditions appears to have selected for reduced aggressiveness, and we show that this renders potential migrant males from extreme habitats less competitive in intrasexual combat when the resident males inhabiting benign habitats show "normal" aggressive behaviour. As the mating system of our study species is based on male dominance hierarchies, with dominant males

aggressively defending small shoals of females from intruders [16–18], we argue that this pattern directly translates into reproductive inferiority of such migrant males.

*1.2. Life in Extreme Habitats.* Habitats can be considered extreme if certain characteristics of the environment are outside of the range normally experienced by a species and if organisms colonizing this particular habitat type experience an initial reduction in fitness [19, 20]. For example, some extreme environments are characterized by exceptionally high concentrations of hydrogen sulphide ($H_2S$): deep-sea hydrothermal vents, hydrocarbon seeps, as well as intertidal zones, salt marshes, mudflats, and sewage outfalls, where $H_2S$ is usually of biogenic origin [21–24]. $H_2S$ inhibits aerobic respiration due to its interference with mitochondrial respiration and blood oxygen transport, but also leads to extreme hypoxia in the water [21, 22]. This makes $H_2S$ acutely toxic to most metazoans even in micromolar amounts, and accordingly, pulses of $H_2S$ discharge have been reported to be the source of mass mortalities [22].

An environmental toxicant like hydrogen sulphide that requires energetically costly behavioural (i.e., actively avoiding microhabitats with high levels of toxicity) and physiological adaptations (various forms of detoxification) by animals exposed to it will certainly have a profound influence on the evolutionary trajectories of populations experiencing the stressor [6]. For instance, when exposed to $H_2S$ and hypoxia, livebearing fishes resort to aquatic surface respiration (ASR) and, thus, exploit the more oxygen-rich air-water interface [25]. Under experimental conditions, Atlantic mollies (*Poecilia mexicana*) have been shown to spend more than 60% of their time performing ASR when exposed to sulphidic water [25], and in natural populations *P. mexicana* have been observed to spend up to 84% of their time performing ASR [26]. However, while access to the water surface (i.e., the possibility to perform ASR) is a strong predictor of short-term survival in fish exposed to $H_2S$-containing water [25], time spent at the water surface clearly trades off against the time fish can spend foraging. Hence, fish from $H_2S$-containing habitats tend to have less food in their guts and lower body condition than conspecifics from nonsulphidic habitats [26–29].

Beside toxicants, perpetual darkness, like in cave ecosystems, can represent an extreme condition for typical surface-dwelling organisms like *P. mexicana* [30]. Darkness renders visual orientation and navigation an impossible task, and cave organisms need to develop specific adaptations to cope with this situation [31–34]. Cave animals (especially crustaceans and fishes) are widely used model organisms to study the evolutionary effects of permanent darkness on various traits, including improved nonvisual sensory systems and increased starvation tolerance (e.g., [34–39]).

*1.3. Ecological Speciation in Extremophile Poeciliid Fishes.* Notwithstanding all the adverse effects of $H_2S$, several species of livebearing fishes (Poeciliidae) have been documented to thrive (and speciate) in waters containing exorbitant concentrations of $H_2S$. Among them are sulphur endemics like the sulphur molly (*Poecilia sulphuraria*) and widemouth gambusia (*Gambusia eurystoma*) [8, 27, 40], as well as species that are currently undergoing ecological speciation, like certain populations of *P. mexicana* [5, 6, 28, 41–43].

Of particular interest are different locally adapted *P. mexicana* populations in the Cueva del Azufre system (Tabasco, Mexico), a system that is characterized by the simultaneous action of two strong selective forces: permanent darkness in subterranean parts of streams and toxic $H_2S$ [6, 30, 44, 45] of volcanic origin [46–48]. Within a small geographic range of only few kilometres, reproductively isolated populations of *P. mexicana* inhabit environments characterized by all possible combinations of these two factors: a toxic cave (Cueva del Azufre, CA), a nontoxic cave (Cueva Luna Azufre, LA), and toxic surface waters; however, a small cascade separates all extreme habitats from nonsulphidic, normoxic sites (for discussion see [42]).

Another system considered in our present study is the sulphur molly system situated at the Baños del Azufre near Teapa (Tabasco, Mexico). This system is characterized by even higher $H_2S$ concentrations (around 230 $\mu$M [8, 43]). Just like in the Cueva del Azufre system, no barriers other than presence of environmental stressors prevent movement of fish among different habitat types in this system [43]. *P. sulphuraria* forms a monophyletic sister clade with phylogenetic affinity to a northern clade of *P. mexicana* rather than *P. mexicana* inhabiting the clear-water habitats in the vicinity of the Baños del Azufre [6]. Thus, sulphur mollies appear to represent a phylogenetically old sulphur-adapted lineage and have been considered a potential "endpoint" of $H_2S$ adaptation [27].

Extremophile *P. mexicana* in the Cueva del Azufre system are characterized by site-specific local adaptations in several behavioural (e.g., [25, 26, 49–51]), dietary [52], female and male life-history [27–29, 53, 54], morphological [17, 43, 45, 55, 56], and physiological traits [17, 57], and there is strong evidence for convergent patterns of $H_2S$ adaptations across both aforementioned sulphur systems [6, 27].

*1.4. Reproductive Isolating Barriers in Extremophile Poeciliids.* Gene flow between populations with different ecological backgrounds in the Cueva del Azufre system is virtually absent with the exception of some degree of genetically detectible migrants from CA found outside of that cave (inside the El Azufre River, EA; [41, 42]). This may be due, in part, to the release of Barbasco, a fish toxicant containing rotenone, during an annual fertility ceremony (La Pesca) of the indigenous Zoque people inside the CA. As Barbasco does not lead to 100% mortality rates, it was suggested that gene flow between the two habitat types may actually be mediated by a certain degree of downstream drift of sedated individuals [58].

Strong reproductive isolation among populations from ecologically divergent habitat types appears to be the result of a combination of natural selection (i.e., direct effects of toxicity, darkness, and predation) and sexual selection through female choice [5, 8, 26, 59], both of which are acting against immigrant individuals. Specifically, $H_2S$ was

shown to be a strong selective force in the aforementioned systems as revealed by reciprocal translocation experiments between nonsulphidic and sulphidic surface habitats [5]. Fish from nonsulphidic habitats had low survival in sulphidic habitats, whereas fish from sulphidic habitats performed poorly under nonsulphidic conditions. Those differences are underlined by tests on $H_2S$ tolerances as fish from sulphidic habitats exhibited consistently higher tolerances than fish from nonsulphidic habitats [6]. The high mortalities of fish in translocations from sulphidic into nonsulphidic environments were hypothesized to be caused by oxidative stress, as oxygen is inherently toxic due to its biotransformation into reactive oxygen species, and organisms have evolved biochemical pathways with antioxidant activity (e.g., superoxide dismutase, catalase, and glutathione systems [60]). During hypoxia, the expression of antioxidant enzymes is often downregulated [61, 62], such that subsequent exposure to normoxic conditions causes substantial oxidative stress with profound fitness consequences [61, 63]. Oxidative stress, possibly in combination with the often poor body condition and energy limitation of fish from sulphidic habitats [25, 27–29, 52], may explain the high mortality seen in migrants from sulphidic to sulphide-free environments.

Contrary to translocations between sulphidic and nonsulphidic habitats, a transfer of fish between sulphidic cave and surface habitats had no effect on survival in either direction. This is not unexpected, as presence or absence of light is unlikely to affect survival within only 24 h. Nevertheless, common garden experiments found that while surface females fail to reproduce in darkness, cave females reared in light are not affected [59]. This is congruent with the aforementioned pattern of unidirectional gene flow from the inside of the caves towards the outside in the Cueva del Azufre system [6, 41, 42]. A further natural selection factor against immigrants was uncovered through similar translocation experiments (outside versus inside cave) that involved the presence of a predator (a giant water bug of the genus *Belostoma*) as heteropterans were more likely to attack cavefish in light but surface fish within the cave [64].

Beside environmental factors acting more or less directly on the viability of migrants in foreign habitats also sexual selection was found to constitute a reproductive isolation barrier. Thus far, only effects of intersexual selection (female mate choice) were assessed. For example, females from the Cueva del Azufre system, including normal surface habitats, sulphidic surface habitats (EA), and the Cueva del Azufre cave (front chambers of CA, which still receive some dim light), discriminate against males from foreign habitats and preferentially associate with males from their own habitat type [5]. Similarly, in the sulphur molly system female *P. mexicana* show strong assortative mating under nonsulphidic conditions, that is, associated less with males of the sulphur-endemic *P. sulphuraria* [8]. Immigrant males from ecologically divergent habitats are consequently at a disadvantage by sexual selection (see also [65, 66]).

In the present paper, we addressed another aspect of sexual selection, namely, intrasexual selection, and asked whether divergent evolution of male aggressive behaviour (i.e., competitive abilities) could play another role in facilitating reproductive isolation among diverging populations by selecting against (maladapted) migrant males. Cave mollies from CA are well known for their reduced aggressiveness [16, 17], and this reduction appears to increase gradually from the entrance to the innermost parts of the cave [67, 68]. When analysing aggressive behaviour with light of various intensities fights occurred at first at 5 lux [69]. When hybrids and backcrosses between cave and epigean fish were tested [68], the frequency of distribution patterns for aggressive fin erection and S-position revealed a genetically based reduction of the aggressive behaviour within CA fish. The $F_1$ generation had an intermediate value for the average, and the variability was practically halved in comparison to the epigean forms. It was concluded that the reduction for aggression is based and controlled by a polygenic genetic system. Furthermore, it was suggested that costly aggressive behaviours lack stabilising selection in darkness where visual perception of an opponent is prohibited; accordingly, reduced aggression was interpreted as a consequence of cave adaptation, that is, evolution under perpetual darkness [17]. Due to the young age of the CA cave molly this reduction process is thought to be still ongoing, eventually leading to the complete reduction of aggressive behaviour in this cave-dwelling population. Despite the extensive work on male aggression in fish from the CA, nothing is known about male aggressive behaviour of *P. mexicana* from the newly discovered sulphide-free Cueva Luna Azufre (LA) which is thought to have been colonized even more recently than the neighbouring CA cave [45]. Moreover, little is known about whether or not presence of toxic $H_2S$ also plays a role for the reduction of aggressive behaviour and, if this was the case, whether evidence for convergent evolution in other drainages containing $H_2S$ can be uncovered. Our hypothesis that not only darkness in caves, but also $H_2S$ might affect the evolution of aggressive behaviour is based on the following considerations. Fish from $H_2S$-containing waters were found to have lower body conditions and fat stores [27–30, 52], most probably due to altered time budgets because of the amount of time being spent in ASR [26] and the physiological cost of $H_2S$ detoxification [22]. These factors have been hypothesized to account for the observed heritable reduction of male sexual activity and sexual harassment of females found in all extremophile populations [50, 70].

In the present study, we asked the following specific questions.

(a) What are the independent and interactive effects of $H_2S$ and darkness on the evolution of aggressive behaviour in the Cueva del Azufre system? Do both stressors ($H_2S$ and darkness) select for reduced aggression? We observed the outcome of dyadic aggressive interactions in male pairs from all divergent populations in the Cueva del Azufre system. For *P. mexicana* ecotypes from this system broad-sense heritability of population differences in the tendency to respond aggressively could be estimated by investigating laboratory- (i.e., common garden-) reared fish.

(b) Is there evidence for convergent evolution (i.e., reduction) of aggressive behaviour in another system

Table 1: Overview of populations used in this study. Given are relevant ecological habitat parameters [light absent (−) or present (+); $H_2S$ absent (−) or present (+)], origin of test individuals [lab-reared (lr) or wild-caught (wc)], as well as coordinates of the sampling sites.

| | Light | Sulphide | Origin | Latitude | Longitude |
|---|---|---|---|---|---|
| Tampico (Tam) | + | − | lr | 22.29632 | −97.90022 |
| Río Oxolotán (Ox) | + | − | lr | 17.44444 | −92.76293 |
| El Azufre (EA) | + | + | lr | 17.44225 | −92.77447 |
| Cueva del Azufre II (CA-II) | − | + | lr | 17.44225 | −92.77447 |
| Cueva del Azufre V (CA-V) | − | + | lr | 17.44225 | −92.77447 |
| Cueva del Azufre X (CA-X) | − | + | lr | 17.44225 | −92.77447 |
| Cueva Luna Azufre (LA) | − | − | lr | 17.44225 | −92.77447 |
| Río Ixtapangajoya (IX) | + | − | wc | 17.49450 | −92.99763 |
| *Poecilia sulphuraria* (PS) | + | + | wc | 17.55225 | −92.99859 |
| Cueva del Azufre II (CA-II) | − | + | wc | 17.44225 | −92.77447 |

with high and sustained $H_2S$, namely, *P. sulphuraria* inhabiting the Baños del Azufre? While fish from the Cueva del Azufre system are easy to maintain and readily reproduce in the laboratory under nonsulphidic light conditions [17, 59], none of our attempts to breed *P. sulphuraria* have been successful so far, as fish would typically die within some weeks upon transfer to the lab. Therefore, for the comparisons among ecotypes in this system we had to rely on wild-caught fish and conducted our experiments on site in Southern Mexico.

(c) Cave-adapted blind characids (*Astyanax mexicanus*) show reduced aggression [71], but were found to increase aggressiveness and to defend small feeding territories when starved [72]. Based on these findings, we asked if *P. mexicana* from CA and EA (i.e., populations showing reduced aggression) would also become more aggressive when starved and thus compared aggressive behaviour of male dyads that had undergone different feeding treatments (high diet versus one week starvation).

(d) Does divergent evolution of aggressive behaviour in extremophile mollies translate into males being inferior in competition with more aggressive males from populations evolving under benign conditions? We simulated a potential migration scenario where the least aggressive CA males were paired with males from a nonsulphidic, normoxic surface stream and investigated their aggressive interactions as well.

## 2. Materials and Methods

*2.1. Study System.* The Atlantic molly, *P. mexicana,* is widespread in freshwater surface habitats along the Atlantic versant of Central America [73]. For our experiments we used both wild-caught fish (experiment 3) and lab-reared descendents of wild-caught fish (all other experiments). Laboratory-reared *P. mexicana* originated from the Río Oxolotán (Ox), a river with mostly clear water in the vicinity of the caves [6, 30], and from the brackish coastal waters near Tampico (Tam; Tamaulipas, eastern Mexico). As representatives from extreme habitats we used descendents from the sulfidic El Azufre (EA), a creek flowing out of the Cueva del Azufre [6, 30]. We furthermore used fish from three distinct cave chambers of the sulphidic Cueva del Azufre (chamber II (CA-II), chamber V (CA-V), and chamber X (CA-X); after [44]) and males from the newly discovered nonsulphidic Luna Azufre cave (LA, [45]). Wild-caught fish for experiment 3 were *P. mexicana* from the nonsulphidic Río Ixtapangajoya (IX, [74]) and from chamber II of the Cueva del Azufre (CA-II), as well as male *P. sulphuraria* (PS) from the Baños del Azufre [40]. GPS coordinates for all sampling localities are given in Table 1.

*2.2. Test Fish and Their Maintenance.* Laboratory stocks were maintained in large, randomly outbred single-species tanks at the Department of Ecology and Evolution of the University of Frankfurt or at the Department of Zoology at the University of Oklahoma in Norman. At both facilities, fish were reared as mixed-sex stocks in 200-L (Frankfurt: Tam, Ox, LA) or 1,000-L tanks (Norman: EA, CA-II, CA-V, CA-X) at 25–27°C under a 12 : 12 hrs light : dark cycle (Frankfurt) or ambient light conditions (Norman) and were fed *ad libitum* at least once daily with commercial flake food. All lab-reared fish were kept under normoxic conditions without $H_2S$, and test fish were descendants of wild-caught fish of the 2nd to 4th laboratory generation.

In experiment 3 we used wild-caught fish, because *P. sulphuraria* could not be maintained under laboratory conditions for more than some weeks, most probably due to their high degree of adaptation to $H_2S$-containing water [6]. Upon capture, fish were transferred into closed and aerated (38 L, 43 × 31 × 32 cm) black Sterilite containers, and we gave them 24 h to acclimate before testing them in a field laboratory as described below.

*2.3. Behavioural Tests*

*2.3.1. General Testing Procedure.* We determined male aggressive behaviours during dyadic encounters by analysing contests staged between pairs of males in a small test tank measuring 30 × 20 ×20 cm. To avoid any confounding effects

of previously established dominance and/or familiarity (see [75, 76]), males of each dyad were taken from different stock tanks. We separated both males by an opaque filter sponge while all sides of the test tank were taped with grey paper to minimize disturbances from the outside. The bottom of the tank was filled with black gravel, and water was kept at 27–29°C and aerated. All experiments were performed with normoxic, nonsulphidic water. Males could habituate to the test tank overnight, and fight observations took place the next day between 09:00 and 13:00. As even size-matched males differed slightly in their fin and general body colouration and were thus easily distinguishable, we noted individual characteristics of both males prior to the fights. At the start of the experiment, the partition separating both males was lifted, and we noted male-male interactions for a maximum of 10 minutes, starting with the first male-male interaction. We focused on three aggressive behaviours that occur frequently in *Poecilia* spp. (after [16]). (1) S-position: this threatening behaviour usually initiates a fight. Males swim in a parallel or antiparallel position and bend their bodies in an S-shaped manner while all unpaired fins are erected. (2) Tail beats: S-positons are often followed or superimposed by tail beats, which are fast moves of head and tail in opposing direction that either touch the opponent's body or send shock waves towards the opponent. (3) Bites: we defined all incidences of ramming and mouth attacks into the direction of the opponent as bites, because these behaviours occur too fast and are too similar to be distinguishable by the human eye.

We also recorded the duration of the fights until dominance was established. Contest outcome was indicated by behavioural differences between the competitors. Folded fins, head-down posture, and a position at the periphery of the tank typically characterized the loser of the contest [77]. Winners, on the other hand, constantly chased and further attacked the losers with spread fins while occasionally performing S-positions. We, therefore, separated both males immediately after dominance was established to avoid serious injuries. If no dominance was established after 10 minutes of observation we terminated the fight and scored fights as "no clear winner"; those trials were discarded from the analysis of fighting durations, while fight durations were scored as "0" when no aggressive behaviour occurred at all. After a contest, body size of all males was measured as standard length (SL) to the nearest millimetre by laying the fish flat on plastic foil-covered millimetre paper, and we transferred the males back into their respective stock tanks. Despite the loss of single scales, no severe injuries and no mortality related to the experiments were observed.

*2.3.2. Experiment 1: When Do Fights Escalate?* The assessment of an opponent's resource holding potential (RHP; see [78]) is crucially connected to the opponents' body size difference in poeciliid fishes [77], and at least in swordtail fish (*Xiphophorus* spp.) fighting intensity (determined as numbers of bites per minute) correlates negatively with the opponents' size difference, but simultaneously was also found to vary greatly when size differences were small [77]. We, therefore, first examined the correlation between fight intensity (determined as numbers of bites per minute) and the opponents' absolute body size (measured as standard length) difference using Spearman's rank order test. We tested 17 male dyads of *P. mexicana* (Tam), while relative size differences within each pair ranged from 0% to 47% (mean pair size: 35.8 ± 1.8 mm). Fight intensity was plotted against opponents' absolute body size difference, and a logarithmic model was used to generate a reference line.

Despite the predicted large degree of variability in fight intensity (see [77]), escalating fights in swordtails (defined as both males biting each other) are more often found when body size differences are small [76, 77]. As the aim of our study was to compare maximum aggressiveness of escalating fights in different *P. mexicana* populations we furthermore tried to estimate the maximum relative opponents' size difference (determined as fraction of standard length the larger male exceeded the smaller male) up to which escalating fights can be observed. To do so, a score expressing how equally both males dedicated their aggressions towards each other in a dyad was calculated as a measure of escalation as: 1 – Abs((sum aggressive behaviours male one − sum aggressive behaviours male two)/sum of aggressive behaviours of male one and male two).

Escalation scores could range between 0 and 1, with values around 0 indicating that only one male showed aggressive behaviour (uneven, no escalated fight) and values around 1 indicating that both males dedicated similar amounts of aggressive behaviours towards each other (even, escalated fight). Scores were plotted against arcsine- (squareroot-) transformed relative body-size differences, and a logistic 4-parameter curve estimation (upper constraint set to 1, lower constraint to 0) was used to determine the $\text{Evenness}_{50}$-score (body size difference at which the score value is 0.5). When opponents' body size differences exceeded the body size difference at the $\text{Evenness}_{50}$-score we assumed fights to be less escalated.

For statistical reasons, scores and body-size differences equal 0 were substituted by 0.001, as logistic models require positive nonzero data.

*2.3.3. Experiment 2: Evolution of Male Aggressiveness in Response to Environmental Stressors.* It was our intention to disentangle the relative effects of sulphur and darkness on the evolution of aggressive behaviour. In a first step we tested whether populations from sites with the same combination of ecological stressors would show comparable levels of aggressive behaviour and, thus, compared the two populations from nonsulphidic surface sites (Tam and Ox) as well as the three CA populations (CA-II, CA-V, CA-X) using similar MANCOVA and ANCOVA models as outlined below. The MANCOVA models with numbers of aggressive behaviours per fight as dependent variables neither detected a significant population difference between both nonsulphidic surface populations ($F_{3,20} = 0.44$; $P = 0.77$) nor between the three CA populations ($F_{6,38} = 1.11$, $P = 0.38$). When comparing fight durations using ANCOVA models we did not find population differences as well (surface: $F_{1,22} = 1.86$, $P = 0.19$; CA: $F_{2,20} = 0.26$, $P = 0.77$).

Based on these results we analysed numbers of aggressive behaviours per fight in seven populations of *P. mexicana* from different ecological backgrounds in our main analysis (see Table 1 for a detailed description of collection sites): A MANCOVA model with "number of S-positions," "number of tail-beats," and "number of bites" as dependent variables and "sulphur" (present/absent) as well as "light" (present/absent) as fixed factors was employed, and "mean pair size" as well as "body size difference" (arcsine (square root) transformed) were included as covariates. We initially included all levels of interaction terms between both main factors and both covariates, but removed interactions from the final model as none of them were significant (not shown). Prior to all analyses, all dependent variables were log transformed and afterwards checked for normal distribution by Kolmogorov-Smirnov tests.

Fighting durations were analysed in a separate ANCOVA model with "sulphur" (present/absent) as well as "light" (present/absent) as fixed factors and "mean pair size" as well as "body size difference" as covariates. No interaction term was significant (not shown), and thus interactions were excluded from the final model.

*2.3.4. Experiment 3: Aggressiveness in the Sulphur Endemic P. sulphuraria.* In this experiment we compared male aggressive interactions among wild-caught individuals of the sulphur endemic *P. sulphuraria* (PS; $N = 9$) and two populations of *P. mexicana,* one from a freshwater surface habitat, the Río Ixtapangajoya (IX; $N = 7$), the other one from the sulphidic Cueva del Azufre (CA-II; $N = 7$ male dyads), in a field laboratory. We used small mice cages (23 $\times$ 15 $\times$ 16.5 cm) instead of our standard test tanks and separated males overnight by opaque plastic sheets. As described for experiment 2, we analysed aggressive behaviours among those three populations through MANCOVA with "mean pair size" as well as "body size difference" as covariates and fight durations in an ANCOVA with "mean pair size" as well as "body size difference" as covariates. In both analysis, interaction terms between the main factor "population" and the covariates were initially included, but removed from the final model as neither had a significant effect.

We used Fisher's LSD tests for pairwise *post hoc* comparison of overall levels of aggression (mean sum of all aggressive interactions per fight) as well as fight durations among populations. In addition, we also compared all three different kinds of aggressive behaviours separately by use of one-way ANOVA and applied Fisher's LSD tests to identify the source of variation when a significant population effect was detected.

*2.3.5. Experiment 4: Aggressiveness and Food Limitation.* As food limitation is predicted to influence the occurrence of aggressive behaviours in fish [79], we compared the intensity of male fights under normal food supply (daily *ad libitum* feeding regime) with fights of males that were subject to a 1-week starvation period. To do so, we separated males from the CA-X and EA populations for 6 days in 50-L tanks and deprived them of food. After this period males that had not been fighting against each other (i.e., stemmed from different tanks) were transferred into our standard test tanks, and fights were observed on the following morning (hence, males starved for 7 days altogether). We analysed numbers of aggressive behaviours per fight (including data for nonstarved males from experiment 2) in a MANCOVA model with "population" (2 levels) and "treatment" (nonstarved/starved) while including "mean pair size" as well as "body size difference" as covariates. Analogously, fight durations were analysed with the same factors and covariates in an ANCOVA model. In both analysis, interaction terms of the main factors "population" and "treatment" and the covariates were initially included but removed from the final model as neither had a significant effect.

*2.3.6. Experiment 5: Male Aggression as Reproductive Isolation Barrier.* Reproductive isolation is crucial for speciation processes, and intrasexual competition may provide one possible mechanism to exclude immigrants from reproducing in foreign habitats. To test this idea, we staged contests between size-matched males from CA (CA-X; mean SL = 30.4 $\pm$ 0.7 mm) versus males from a sulphide-free surface habitats (Ox population; 30.7 $\pm$ 0.8; paired $t$-test on size differences: $t_{12} = -0.81$; $P = 0.45$). Chi$^2$ tests were employed to compare numbers of fights won by males from either population, and numbers of aggressive behaviours shown by the two ecotypes within each male dyad were analysed using paired $t$-tests. We further recorded and compared all sexual behaviours (nipping and copulation attempts, so-called thrusting; see [16] for a description) between both male types, as cave mollies may answer aggressions by sexually motivated behaviours [68].

## 3. Results

*3.1. Body Size Difference between Opponents and Male Aggressive Behaviour.* In our first experiment we quantified fight intensities and durations in staged contests of *P. mexicana* males from the Tampico population. Body size differences between both males within a dyad varied between 0 and 8 mm. Fight intensity (measured as bites per minute) was negatively correlated with the opponents' body size difference (Spearman rank order test; $r_s = -0.52$, $P = 0.033$; Figure 1(a)) meaning that males fought most intensely when both opponents were closely size matched. The body size difference below which fights escalated (i.e., below which both males displayed equal numbers of aggressive behaviours; "fight evenness") was determined as 7.7%, with the 95% confidence interval ranging between 5.1% and 12.2% (Figure 1(b); Logistic model: $R^2 = 0.51$, $F_{1,16} = 15.79$). Based on these results, we made an attempt to use closely size-matched male pairs in all subsequent experiments [mean ($\pm$SD) size difference = 5.4 $\pm$ 8.2%] and included arcsine- (square root-) transformed relative body size difference of each dyad as a covariate in all further analyses.

*3.2. Evolution of Male Aggressiveness in the Cueva del Azufre System.* MANCOVA revealed a significant effect of the factor

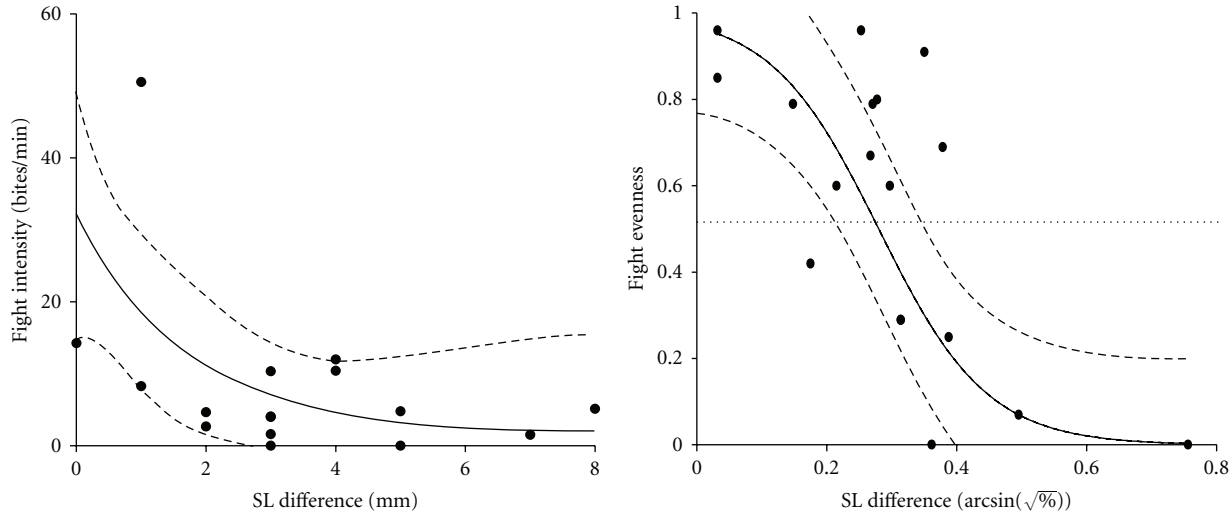


Figure 1: (a) Fight intensity and (b) "fight evenness" (see main text) in relation to the two opponents' body size difference (as standard length (SL) difference). Shown are regression lines representing the best-fit (a: logarithmic model; b: logistic model) and 95% confidence intervals ($N = 17$ fights).

Table 2: Results from (a) MANCOVA and (b) ANCOVA models analysing attributes of dyadic male aggressive interactions in Experiment 2 (lab-reared males). $F$-ratios were approximated using Wilk's $\lambda$. Partial variance was estimated using Wilk's partial $\eta^2$. Significant effects are in bold typeface.

| | df | $F$ | $P$ | Partial variance explained [%] |
|---|---|---|---|---|
| (a) MANCOVA (number of aggressive behaviours) | | | | |
| Light (absent/present) | **3,62** | **8.97** | **<0.001** | **0.30** |
| Sulphide (absent/present) | 3,62 | 2.45 | 0.072 | 0.10 |
| Light × sulphide | **3,62** | **3.77** | **0.015** | **0.15** |
| Male body size difference | **3,62** | **3.35** | **0.025** | **0.14** |
| Mean pair body size | **3,62** | **2.81** | **0.044** | **0.12** |
| (b) ANCOVA (fight duration) | | | | |
| Light (absent/present) | **1** | **8.44** | **0.005** | **0.12** |
| Sulphide (absent/present) | **1** | **7.07** | **0.010** | **0.10** |
| Light × sulphide | 1 | 2.48 | 0.120 | 0.04 |
| Male body size difference | 1 | 0.25 | 0.622 | <0.01 |
| Mean pair body size | 1 | 0.71 | 0.403 | 0.01 |
| Error | 64 | | | |

"light" (Table 2(a)), indicating that cave-dwelling populations displayed significantly fewer aggressive behaviours than surface fish (Figure 2(a)). The significant "light × sulphide" interaction (Table 2(a)) further indicates that this reduction in aggressiveness is aggravated in populations evolving under both extreme conditions, while "sulphur" *per se* did not lead to a significant reduction in aggressive behaviours (Table 2(a); Figure 2(a)). Also both covariates ("mean opponent body size" and "body size difference") had a significant influence in our model (Table 2(a)), and *post hoc* Spearman rank-order tests revealed that "mean opponent body size" was positively correlated with numbers of S-positions ($r_s = 0.32$, $P = 0.007$), tail beats ($r_s = 0.30$; $P = 0.013$), and bites per fight ($r_s = 0.44$; $P = 0.001$), suggesting that fights of larger males were more intense than those of smaller ones. In contrast, the body size difference between both opponents was negatively correlated with the number of S-positions ($r_s = -0.34$; $P = 0.004$) and tail beats ($r_s = -0.24$; $P = 0.043$), but not fights ($r_s = -0.19$; $P = 0.12$), indicating that the larger the opponents' body size difference was, the less intense fights became.

When comparing the duration of fights we found both main factors ("light" and "sulphide") to have significant effects (Table 2(b)). This and the nonsignificant interaction term of "light × sulphide" suggest that both the absence of light and the presence of $H_2S$ lead to similar reductions in fighting time (Figure 2(b)).

*3.3. Aggressiveness in the Sulphur Endemic P. sulphuraria.* When comparing numbers of aggressive behaviours in fights

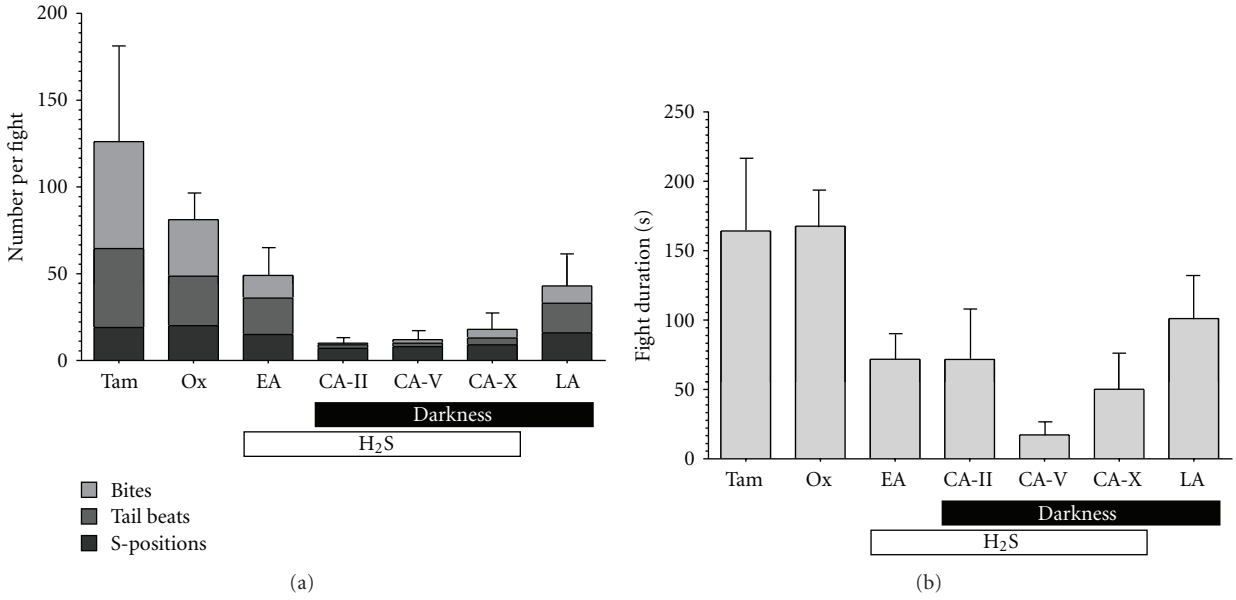


Figure 2: Means (±SE) of (a) numbers of aggressive interactions and (b) fight duration in seven populations of *P. mexicana*. From left to right: populations from nonsulphidic surface sites [Tampico, Tam ($N = 12$), and Río Oxolotán, Ox ($N = 14$)], the sulphidic creek in the Cueva del Azufre system [El Azufre, EA ($N = 9$)], three cave chambers of the sulphidic Cueva del Azufre [CA-II ($N = 12$), CA-V ($N = 7$), CA-X ($N = 6$)], and the $H_2S$-free cave [Luna Azufre, LA ($N = 10$)].

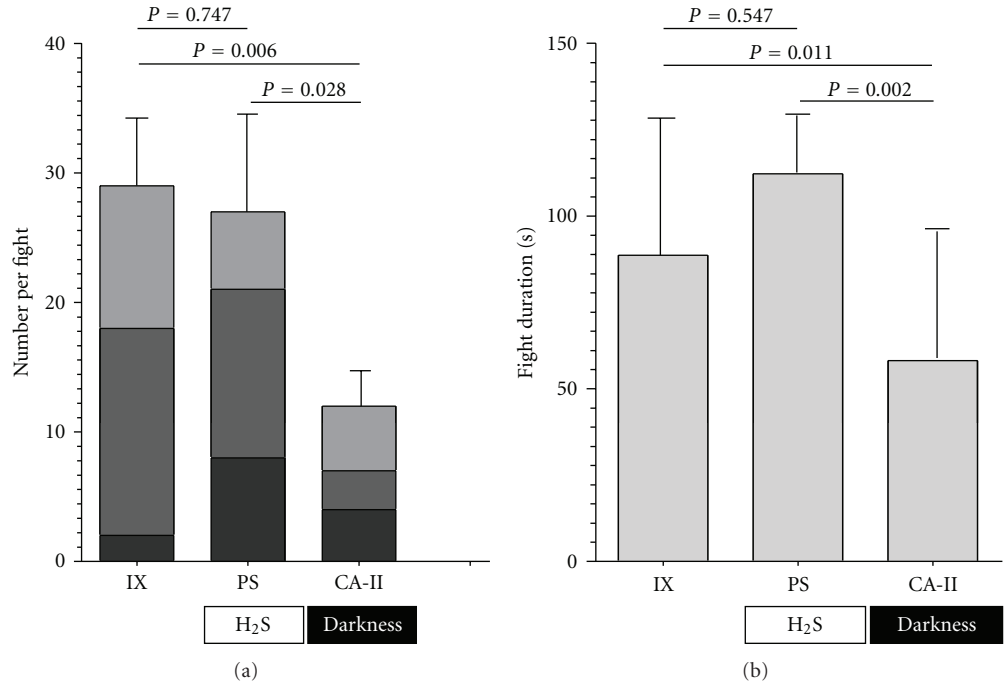


Figure 3: Means (±SE) of (a) numbers of aggressive behaviours shown by males during dyadic fights and (b) duration of fights in wild-caught males from two *P. mexicana* populations [the sulphide-free Río Ixtapangajoya, IX ($N = 9$) and cave chamber II of the sulphidic Cueva del Azufre, CA-II ($N = 7$)], as well as the sulphur-endemic *P. sulphuraria* (PS) found at the Baños del Azufre ($N = 7$).

of wild-caught males from two *P. mexicana* populations (IX and CA-II) and *P. sulphuraria* males by use of MANCOVA we found a significant effect of the factor "population/species" ($F_{6,32} = 3.54$; $P = 0.009$), and *post hoc* pairwise comparisons (Fisher's LSD) showed levels of aggressive behaviours of surface *P. mexicana* and *P. sulphuraria* to differ significantly from those seen in *P. mexicana* males from CA-II (Figure 3(a)). None of the covariates had a significant effect (male body size difference: $F_{3,32} = 1.24$; $P = 0.322$; mean pair body size: $F_{3,32} = 0.44$; $P = 0.722$). One-way ANOVAs

confirmed significant differences between populations in all three aggressive behaviours (S-position: $F_{2,20} = 4.28$, $P = 0.028$; tail beats: $F_{2,20} = 7.51$, $P = 0.004$; bites: $F_{2,20} = 10.98$, $P = 0.001$). *Post hoc* tests revealed that fights between *P. sulphuraria* males were characterized by significantly more S-positions compared to fights of IX males ($P = 0.008$), and fights of both surface populations/species displayed significantly more tail beats compared to fights of CA-II males (IX versus CA-II: $P = 0.001$; PS versus CA-II: $P = 0.007$). All three populations differed significantly in numbers of bites per fight (IX versus CA-II: $P < 0.001$; IX versus PS: $P = 0.045$; PS versus CA-II: $P = 0.011$).

When analysing the durations of fights, our ANCOVA model detected a significant effect of the factor "population/species" ($F_{2,18} = 5.59$; $P = 0.013$), and pairwise comparisons showed that both surface forms (IX and PS) fought significantly longer than *P. mexicana* males from CA-II (Figure 3(b)). Again, both covariates were not significant (male body size difference: $F_{1,18} = 0.09$; $P = 0.763$; mean pair body size: $F_{1,18} = 0.02$; $P = 0.886$).

*3.4. Aggressiveness and Food Limitation.* When comparing numbers of aggressive behaviours in fights of *P. mexicana* from CA-X and EA under normal food supply (data from Experiment 2) and after one week of starvation in a MANCOVA we found a significant effect of the factor "food treatment" ($F_{3,29} = 3.68$; $P = 0.023$) while the factor "population" ($F_{3,29} = 1.31$; $P = 0.29$) as well as the interaction term "treatment × population" was not significant ($F_{3,29} = 0.48$; $P = 0.70$). This indicates that both populations reduced their aggressive behaviour in a similar fashion when food was scarce (Figure 4(a)).

Like in experiment 2, we found the covariate "mean opponent size" to have a significant effect in the MANCOVA ($F_{3,29} = 3.28$; $P = 0.035$), and *post hoc* Spearman rank-order tests revealed a significant positive correlation between "mean opponent size" and numbers of S-positions ($r_s = 0.46$; $P = 0.004$) and tail beats ($r_s = 0.43$; $P = 0.007$), but not bites ($r_s = 0.35$; $P = 0.13$). The covariate "body size difference" had no significant effect ($F_{3,29} = 1.28$; $P = 0.30$).

Another ANCOVA model analysing fighting durations revealed a significant effect of the factor "food treatment" ($F_{1,31} = 4.44$; $P = 0.043$) while the factor "population" ($F_{1,31} = 0.02$; $P = 0.90$) and the interaction term "treatment × population" were not significant ($F_{1,31} = 1.76$; $P = 0.19$). Furthermore, both covariates had no significant effects ("body size difference": $F_{1,31} = 1.10$; $P = 0.30$; "mean opponent size": $F_{1,31} = 2.51$; $P = 0.12$). Starvation in general reduced the duration of fights (Figure 4(b)).

*3.5. Fights between Different Locally Adapted Males.* In all 13 staged contests, Río Oxolotán (Ox) males established dominance over the CA-X males ($\text{Chi}^2 = 13.0$, df = 1, $P < 0.01$) after a mean fight duration of 119 ± 19 s. Ox males directed significantly more aggressive behaviours towards CA-X males (S-position: $t_{12} = -4.12$, $P = 0.001$; tail-beats: $t_{12} = -4.50$, $P < 0.001$; bites: $t_{12} = -5.38$, $P < 0.001$; Figure 5) while cave molly males directed more sexually motivated behaviours towards Ox males during the fights (nipping: $t_{12} = 4.49$, $P < 0.001$; thrusting: $t_{12} = 3.43$, $P = 0.005$; Figure 5).

## 4. Discussion

An increasing body of literature documents adaptation's potential to drive genetic differentiation and ultimately speciation (e.g., [80–83]), a phenomenon that has recently been termed "isolation by adaptation" [84]. Of particular interest in the study of ecological speciation are the proximate mechanisms leading to and maintaining genetic differentiation among populations [2, 84]. During ecological speciation, prezygotic isolation may arise when immigrants from foreign, ecologically divergent habitats are selected against [85, 86]. This may occur through natural selection, if immigrants (or hybrids) have reduced viability (extrinsic reproductive isolation; e.g., [5, 6, 87]), or by sexual selection, if maladapted individuals are discriminated against during mate choice (e.g., [88]).

In the present study we examined whether—in addition to mate choice (i.e., intersexual selection)—intrasexual selection through male-male competition could also play a role in promoting prezygotic isolation. Atlantic molly males in clear-water habitats usually establish dominance hierarchies, and dominant (typically the largest) males monopolize several females which they aggressively defend against rivals [16]. This view is supported by our present findings in that fighting intensity was positively correlated with the average body size of male dyads; in other words, larger males fought more intensely, probably driven by the prospect of monopolizing females. Smaller males, by contrast, rely on a sneak-like mating tactic [50, 70], but such "alternative" mating tactics are lost in extremophile *P. mexicana* [50], likely owing to very similar counterselection in energy-limited habitats that, as we will discuss, may have played a role for the evolutionary reduction of aggressive behaviour (see below).

We found fight intensity to be reduced in various extremophile *P. mexicana* populations, and perpetual darkness in caves was the best predictor for the evolutionary reduction of aggressiveness, especially when it was combined with presence of $H_2S$, as seen in the CA cave. As lab-reared fish were used for this part of our study, the observed differences seem to be largely evolved (genetic) differences among ecotypes. When we considered fight durations, also a significant main effect of the factor "$H_2S$" was observed; fish from sulphidic habitats engage in shorter fights as an evolutionary response to the toxicant. Finally, we demonstrate that reduced aggression directly translates into males being inferior in contests, as evidenced by the fact that Ox males always won when paired with cave molly (CA) males; CA males even responded sexually to aggressive attacks, an obviously maladaptive behaviour (see also [68]). We argue that in a system where dominance hierarchies play a vital role, reduced aggressiveness translates directly into male inferiority in mate competition upon encounter of different behavioural phenotypes. Specifically, we argue that migrant males stemming from an ecological background that has selected for

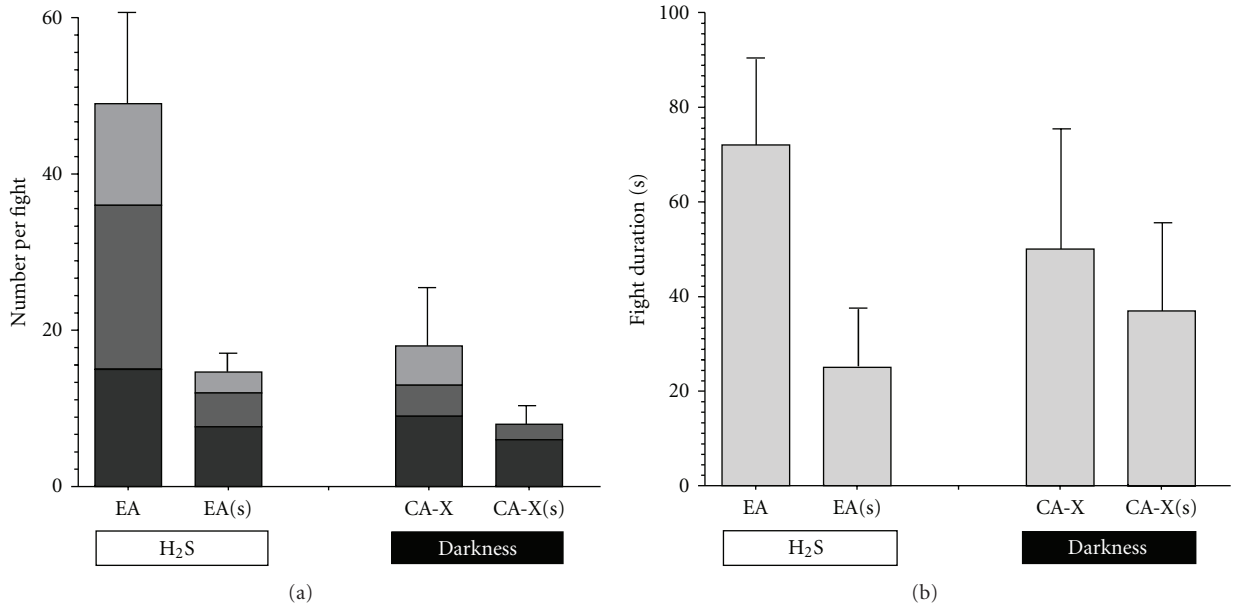


Figure 4: Means (±SE) of (a) numbers of aggressive behaviours and (b) the duration of male fights in males from two extremophile populations in the Cueva del Azufre system, one from the sulphidic surface stream [El Azufre, EA ($N = 21$)] and one from chamber X of the sulphidic Cueva del Azufre [CA-X ($N = 16$)]. Prior to the tests, males were either fed on a normal diet (left bars) or starved for one week (s: right bars).

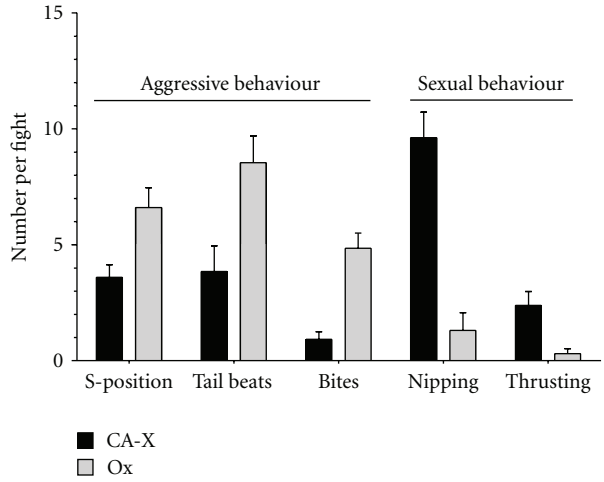


Figure 5: Mean (±SE) numbers of aggressive and sexual behaviours shown by males in cross-population dyadic fights ($N = 13$) between Río Oxolotán (Ox) males and males from chamber X of the sulphidic Cueva del Azufre (CA-X). All comparisons were statistically different between populations (see main text).

reduced aggressiveness may be selected against (i.e., have low reproductive fitness) in a divergent (i.e., benign) habitat type. Together with the action of natural selection against (maladapted) migrants via $H_2S$-toxicity, darkness and predation [5, 6, 8, 26, 59], as well as female mate discrimination against alien male phenotypes [5, 8], divergent evolution of aggressive behaviour may thus play an important role for the maintenance of genetic differentiation in this system—at least at the interface between extreme and benign (nonsulphidic surface) habitats and, hence, could represent another mechanistic link explaining the surprising small-scale genetic structuring in the CA system [41, 42]. It remains to be determined in future studies whether the comparatively small differences in the intensity of aggressive behaviour seen in males from some habitats that are directly adjoining in the Cueva del Azufre system (e.g., CA versus EA, LA versus EA) lead to an equally clearcut picture, that is, if also in those cases it is always the more aggressive males that win a combat. For practical reasons, our present study focussed on aggressive interactions between the most extreme behavioural phenotypes: males from the most aggressive (Ox) population and the least aggressive CA-X males.

Parzefall first described reduced aggression in the CA cave population of *P. mexicana* (from the rearmost chamber XIII; CA-XIII) [16] and interpreted his findings as an adaptation to perpetual lightless conditions [17], as most aggressive behaviours depend on visual perception of cues from opponents, which may be more difficult to perceive in darkness. Even though theory predicts a reduction of intraspecific aggression in troglobites [89, 90], some cave dwellers may even have evolved entire novel sets of aggressive behaviours while responding to nonvisual signals. For example, aggressive behaviour is well developed in the blind catfish *Uegitglanis zammaranoi* [91] and the blind cave salamander *Proteus anguinus* [92]. Furthermore, the discovery of a highly aggressive cave-dwelling *Astyanax fasciatus* population [93] implies that a reduction of aggressive behaviour is not an inevitable evolutionary response to the cave environment. Those authors suggested that explanations other than simply the inability to perceive visual cues triggering aggressive behaviour should be explored in order to explain the evolutionary reduction of aggressiveness in many other *Astyanax* cave fish populations [94, 95].

As we have argued above, previous studies have demonstrated that *P. mexicana* inside the two caves (CA and LA), as well as the toxic surface habitat (EA), appear to be energy limited, as evidenced by their lower body conditions and reduced fat stores [27–30, 52]. In the nontoxic LA cave, this is probably due to low resource availability, which is typical for most caves (reviewed in [37, 96]). In contrast, CA and EA are energy-rich habitats due to high chemoautotrophic primary productivity [97, 98]; however, *P. mexicana* spend the majority of their time at the water surface engaged in ASR [26] and probably pay a high physiological cost in order to run ATP-expensive $H_2S$ detoxification [22]. Not surprisingly, a recent study therefore found cave mollies from CA to have higher metabolic rates compared to surface mollies even after several generations in the laboratory [99]. Altogether, this suggests that reduced aggression is most likely an evolutionary response to continued energy limitation in the Cueva del Azufre system. This hypothesis is corroborated by the results from this study, in which we found that *P. mexicana* from EA and CA plastically reduce their aggression even further after being starved for one week.

It is further interesting to note that not only did overall levels of aggression diverge between extremophile and nonextremophile poeciliids, but also the relative contribution of specific aggressive behaviours to the aggressive repertoire of these species. The potentially most harmful aggressive behaviour (i.e., bites and rammings) was strongly reduced in all extremophile poeciliids, while the least harmful behaviour (i.e., S-positions) was actually increased in extremophiles. As the energetic costs of threat displays were found to be low relative to the costs of escalated fighting in an African cichlid species (*Tilapia zillii*) [100] we argue that again this phenomenon is a response to the energy limitation experienced in extreme habitats. Moreover, *P. mexicana* males appear to have higher energy expenditure than females [25, 101] and, therefore, exhibit higher mortality rates under stressful conditions and perform more ASR than females [25]. Assuming that male poeciliids in $H_2S$-toxic habitats live near the edge of survivability [25], any injuries obtained during fights with other males (especially during biting or ramming) could indeed lead to life-threatening infections and, ultimately, premature death—a hypothesis that is further supported by a recent study reporting on higher individual parasitization rates of *P. mexicana* in the CA and EA compared to Ox [102].

In stark contrast to the findings from the Cueva del Azufre system, our experiments using wild-caught males from another system with high and sustained $H_2S$, namely, *P. sulphuraria* inhabiting the Baños del Azufre, found no significant difference among *P. mexicana* from nonsulphidic sites and the "sulphur-endemic" *P. sulphuraria*. So, why did extremophile males from the Cueva del Azufre system show strongly reduced aggressiveness, but *P. sulphuraria* did not? We propose three mutuallynot exclusive hypotheses. First, our analysis of fish in the Cueva del Azufre system found the relative contribution of the factor "sulphide" to the evolutionary reduction of aggressive behaviour to be much lower than that of the factor "light" (see partial variance explained in Table 2), so these fish may just not experience the same selective pressure to reduce aggressiveness. Second, *P. sulphuraria* are clearly well adapted to high concentrations of $H_2S$ (i.e., being sulphur endemics) and accordingly could show some kind of "rebound effect", indicating evolved mechanisms to better cope with the toxicant (see [20] for discussion). Some support for the latter idea was also found in life-history traits, as *P. sulphuraria* actually had the largest (not the smallest) fat stores in a comparison of poeciliids from several benign and sulphidic habitats [27]. Third, contrary to *P. mexicana* from the Cueva del Azufre system, which are the only permanent piscine residents in their respective extreme habitats [43], *P. sulphuraria* have to share their habitat with another sulphide-adapted species, the widemouth gambusia, *Gambusia eurystoma* [40]. Hence, increased aggression could also be a signal of interspecific competition for resources at the Baños del Azufre.

In conclusion, sulphuric waters are characterized by reduced resource availability but increased energy expenditure, leading to low body conditions and fat stores in $H_2S$-inhabiting *P. mexicana*. We suggest that in addition to darkness in caves also resource limitation might play a crucial role in the evolutionary reduction of male aggressive behaviour. Selection against costly behaviours (such as aggression) might lead sulphur-adapted mollies to trade-off aggressive behaviour to compensate for the negative effects of $H_2S$—similar to the proposed processes resulting in the observed heritable reduction of male sexual activity and harassment of females found in all extremophile populations (e.g., [50]), as well as patterns of life history divergence [28, 29]. On the other hand, the phylogenetically old "sulphur specialist" *P. sulphuraria*, which we did not find to show reduced aggression, might cope better with $H_2S$ and thus can afford to express costly aggressive behaviour.

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*Research Article*

# Rapid Evolution of Assortative Fertilization between Recently Allopatric Species of *Drosophila*

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The virilis group of *Drosophila* represents a relatively unexplored but potentially useful model to investigate the genetics of speciation. Good resolution of phylogenetic relationships and the ability to obtain fertile hybrid offspring make the group especially promising for analysis of genetic changes underlying reproductive isolation separate from hybrid sterility and inviability. Phylogenetic analyses reveal a close relationship between the sister species, *Drosophila americana* and *D. novamexicana*, yet excepting their contemporary allopatric distributions, factors that contribute to reproductive isolation between this species pair remain uncharacterized. A previous report has shown reduced progeny numbers in laboratory crosses between the two species, especially when female *D. novamexicana* are crossed with male *D. americana*. We show that the hatch rate of eggs produced from heterospecific matings is reduced relative to conspecific matings. Failure of eggs to hatch, and consequent reduction in hybrid progeny number, is caused by low fertilization success of heterospecific sperm, thus representing a postmating, prezygotic incompatibility. Following insemination, storage and motility of heterospecific sperm is visibly compromised in female *D. novamexicana*. Our results provide evidence for a mechanism of reproductive isolation that is seldom reported for *Drosophila* species, and indicate the rapid evolution of postmating, prezygotic reproductive barriers in allopatry.

## 1. Introduction

One of the main goals in recent studies of speciation has been to identify the underlying genetic components of reproductive isolation and the evolutionary forces that cause their divergence [1]. Studies of speciation in *Drosophila*, with particular emphasis on the *melanogaster* and *pseudoobscura* species complexes, have begun to reveal the genetics of postzygotic reproductive barriers such as hybrid sterility and hybrid inviability [2–6]. Other forms of reproductive isolation, such as those acting after copulation and before successful fertilization of the egg, have received less attention in studies of *Drosophila* species. The incidence of this form of isolating barrier is hitherto unknown in the genus *Drosophila*; however, accumulating evidence indicates that postmating, prezygotic isolation is strong in select subgroups, such as the *virilis* group [7, 8] where postzygotic barriers are weak.

The ability to obtain fertile hybrid progeny from laboratory crosses between widely divergent species makes the *virilis* species group a particularly good system to investigate mechanisms of reproductive isolation other than hybrid sterility and inviability [7, 9–11]. Informed by molecular phylogenetic analyses of species within the virilis group [12–14], speciation studies have the potential to elucidate the temporal accumulation of reproductive incompatibilities associated with increased divergence. The closely related species pair *D. americana* and *D. novamexicana* is particularly relevant to the study of the initial incompatibilities that accumulate following geographic isolation. Contemporary geographic distributions of these species are separated, respectively, east and west of the Rocky Mountain Range, with isolation estimated to have occurred ~0.4 mya [14]. These allopatric distributions were established in North America following colonization from Eurasia, where a much older (4.5 mya) divergence occurred with *D. virilis*.

Previous studies have primarily investigated reproductive incompatibilities involving *D. virilis* [8, 15, 16], while incompatibilities between the more recently diverged species pair, *D. americana* and *D. novamexicana*, have not been studied beyond the early experiments of Patterson and Stone [17].

While *D. americana* and *D. novamexicana* exhibit clear morphological divergence [18–20], studies of genetic differentiation reveal conflicting patterns depending on the genomic regions examined [14, 17, 21–25]. At the chromosomal level, the species differ by several rearrangements. A fixed centromeric fusion between two autosomes (chromosomes 2 and 3) is present in *D. americana*. Another centromeric fusion between the X and 4th chromosomes is also unique to *D. americana*, but this rearrangement still segregates with the ancestral chromosome forms and exhibits a strong latitudinal cline [26, 27]. Historically, this rearrangement has been used to differentiate northern and southern subspecies of *D. americana* [11, 28]. In contrast to the ancestral arrangement of chromosome arms maintained by *D. novamexicana*, several derived inversions have been fixed, yet in each case both the ancestral and derived arrangements continue to segregate in *D. americana* [29]. These chromosomal rearrangements with their associated sequence variants have the potential to influence patterns of genetic differentiation locally throughout the genomes of these species.

Genetic diversity and differentiation of these sister species appear to be influenced by their distinct demographic histories and the sorting of ancestral variation during the short time since their isolation. Both species occupy riparian habitats; however, *D. americana* is broadly distributed in the mesic environs of the central and eastern USA, whereas *D. novamexicana* is only known from isolated localities in the xeric environs of the southwest USA [11]. Different effective population sizes are evident from the high sequence diversities maintained within nuclear and mitochondrial genomes of *D. americana* compared to the essentially invariant *D. novamexicana* [14, 22, 23, 25]. Phylogenetic analyses of individual loci mostly recover single, but sometimes multiple, monophyletic groups of alleles from *D. novamexicana* embedded within diverse alleles of *D. americana*. This paraphyly indicates that a large portion of the variation currently segregating in *D. americana* is ancestral to the divergence between this species pair, and by contrast this ancestral variation has been either fixed or lost in *D. novamexicana*. Genomic regions are variably related between species, with shared chromosomal rearrangements having localized affects on relationships, but phylogenetic analysis of combined data from multiple genes successfully resolves reciprocally monophyletic clades of the sister species [25]. Overall, these observations are consistent with a scenario wherein, following the initial peripatric split within the *D. americana-D. novamexicana* ancestral population, a much smaller population of *D. novamexicana* persisted west of the Rocky Mountains compared with the broadly distributed populations of *D. americana* to the east.

Given the recent divergence between *D. americana* and *D. novamexicana*, and their close genetic relationship, studies of laboratory crosses are needed to reveal barriers to reproduction that have arisen in the short interval since their isolation, thereby shedding light on the initial mechanisms contributing to the reproductive isolation between species. An investigation of mating preference among members of the virilis group by Spieth [30] suggested that behavioral isolation has not evolved between this species pair. In contrast, Patterson and Stone [10] report asymmetric reductions in the numbers of progeny produced from interspecific crosses; female *D. novamexicana* produce fewer heterospecific progeny than female *D. americana*. Here we investigate the causes of the reduced number of hybrid progeny. We specifically examine the stage of reproduction where incompatibility is expressed, and further investigate the consequences of double inseminations for conspecific and heterospecific crosses. We show that incomplete, noncompetitive gametic isolation is the primary reproductive barrier that has evolved between these species.

## 2. Methods

*2.1. Fly Stocks and Crosses.* All isofemale lines of *Drosophila americana* were derived from flies collected in 1997 or 1999 and are maintained at the University of Iowa. Last two digits of the collection year were used in the line identification. The *D. novamexicana* line (15010-1031.4 from Moab, Utah, USA) was obtained from the Drosophila Species Stock Center (Tucson, AZ). All lines were cultured on standard cornmeal medium at 22°C and with a 14 : 10 light : dark cycle.

Offspring production in conspecific and heterospecific crosses was assessed by collecting virgin flies of *D. americana* (NN97.4-red, from Niobrara, Nebraska, USA) and of *D. novamexicana* (1031.4) 24–48 hours after eclosion and aging in groups of 20–25 for 10 days. Sexually mature individual females were paired with sexually mature individual males in a yeasted food vial until mating was observed and copulation was complete. Males were aspirated from the vial and females were left to lay eggs at 22°C. Adult flies eclosed ≥21 days later and the total number of progeny produced by each female was recorded.

Double matings followed the same regime of virgin collection and aging; however, successful copulation with the first male was followed by pairing and copulation with a second male on the following day. Progeny of each doubly mated female were visibly identified as either conspecific or hybrid. In crosses involving *D. novamexicana* females, hybrids are distinctly darker in body color than conspecific offspring (i.e., due to dominance of the darker pigmentation of *D. americana*). On the other hand, hybrids produced in crosses involving *D. americana* females of the NN97.4-red line, which have a recessive eye-color mutation, exhibit wild-type eye color compared to conspecific homozygotes with mutant red eye color. These data were tested for equality in mean number of progeny using a two-sample $t$-test (JMP 8).

*2.2. Egg Hatch in Conspecific and Heterospecific Crosses.* Egg hatch in conspecific and heterospecific crosses of *D. americana* (NN97.4-red) and *D. novamexicana* (1031.4) was measured through large-scale egg collections. Males and females of the parental lines were isolated as virgins and aged

10 days. Sexually mature adults were mated in groups of 10–20 flies in yeasted cornmeal vials for 3-4 days and introduced into population cages (25 cm × 25 cm × 40 cm) at a density of approximately 200 males and 200 females per cage. Cages were supplied each day with a water source and a new grape juice agar plate containing a dollop of yeast paste. Two days after introducing flies into the cage, 100 eggs were collected daily and arrayed on a new grape juice plate over 10 days from each cross ($n \approx 1000$ for each cross). Numbers of hatched and unhatched eggs were recorded two days after arraying each egg collection.

Variability in conspecific and heterospecific egg hatch was investigated using five additional iso-female lines (HI99.14, PM99.32, LA99.46, FP99.4, and ML97.5) of *D. americana* derived from broadly separated localities distributed throughout the Mississippi River Valley. In addition to measurements of egg hatch for each line in reciprocal heterospecific crosses with *D. novamexicana* (1031.4), conspecific crosses between lines were used to measure egg hatch between geographically separated populations of *D. americana*. Similar procedures for collecting, arraying, and determining egg hatch from population cages were used. A total of 20 heterospecific crosses between *D. americana* lines were performed. Mean heterospecific hatch rate estimates were compared to conspecific hatch rate estimates using the Tukey-Kramer HSD test (JMP 8).

### 2.3. Reproductive Incompatibility Assay

*2.3.1. Fertilization.* The ability of conspecific and heterospecific sperm to successfully fertilize eggs was assessed using a sperm-tail specific rat polyclonal antibody, $\alpha$-XT (provided by Tim Karr, Arizona State University), to visualize the sperm tail within eggs after laying. The two lines used for this experiment were *D. americana* NN97.4-red and *D. novamexicana* 1031.4. Eggs were collected in 30-minute intervals (to ensure eggs were obtained shortly after deposition) from conspecific and heterospecific cage populations until ≥100 were obtained for each cross. Eggs were dechorionated, fixed, and rehydrated as described in [31]. Rehydrated embryos were incubated in a 1 : 300 $\alpha$-XT: PBTA solution for 1 hour, rinsed multiple times, and washed in PBTA overnight at 4°C. Eggs were labeled with Alexa Fluor green fluorescent anti-rat secondary antibody at a 1 : 400 dilution for 1 hour before rinsing extensively (>10 times) and washing in PBTA overnight at 4°C. The rinse/wash cycle was repeated three times. Eggs were rinsed with PBS-Azide before mounting on a slide with 90% Glycerol and observing under 40x magnifications with fluorescence illumination (Leica DM2000). The proportion of eggs containing fluorescently labeled sperm tail was recorded for each of the conspecific and heterospecific crosses. Independent samples of eggs were collected from the same population cages and arrayed on grape juice agar plates to assess the hatch rate.

*2.3.2. Sperm Storage and Motility.* Two different experimental regimes were used to assess the efficiency of storage and motility of sperm in conspecific and heterospecific crosses involving *D. novamexicana* females, where the greatest difference in egg hatch is observed. First, *D. novamexicana* females were mated to an individual conspecific ($n = 26$) or an individual heterospecific male ($n = 24$). Subsets of females from each of the two crosses were dissected 1, 2, and 3 days after copulation. All dissections included only the reproductive organs, which were placed on a slide containing Ringer's solution and overlaid with a cover slip. Sperm motility was qualitatively assessed by phase microscopy where motile sperm appear as a bundle of colored wave-like lines.

A second experimental strategy involved transferring *D. novamexicana* females to 2% agar immediately following mating with either conspecific or heterospecific males. The expectation is that maintenance on 2% agar would reduce the propensity for laying eggs (see Section 3). The number of eggs laid into the agar by each female was recorded after one week, when the reproductive organs were dissected and examined under a light microscope for the presence of motile sperm. Dissections in which any of the sperm storage organs were severed or ruptured were discarded. The presence or absence of sperm in either the spermathecae or seminal receptacle was recorded for each female. Motility was assigned for each of the two storage organs separately on the basis of whether the stored sperm mass displayed rhythmic oscillating motion.

## 3. Results

*3.1. Progeny Numbers in Conspecific and Heterospecific Crosses.* Consistent with results previously reported by Patterson and Stone [10], progeny numbers are reduced in interspecies crosses between *D. americana* and *D. novamexicana* relative to crosses within species (Table 1). The number of progeny produced by female *D. novamexicana* mated to heterospecific males is only about 2% of the number produced by female *D. novamexicana* mated to conspecific males (Table 1; $t = 20.0$, $d.f. = 48$, $P < 0.001$). Progeny number is not reduced as dramatically in heterospecific crosses involving female *D. americana*. In this case, female *D. americana* mated with heterospecific males produce about 30% of the progeny number of females mated with conspecific males (Table 1; $t = 10.7$, $d.f. = 44$, $P < 0.001$). Females of the two species produce similar progeny numbers when mated with conspecific males ($t = -2.02$, $d.f. = 46$, $P > 0.05$) whereas heterospecific crosses with each female *D. novamexicana* produce on average only a single individual, which is significantly less than the number produced by female *D. americana* in heterospecific crosses ($t = -13.02$, $d.f. = 48$, $P < 0.05$).

*3.2. Egg Hatch in Conspecific and Heterospecific Crosses.* Failure of early postcopulatory events was investigated as the cause of reduced progeny numbers in crosses between *D. americana* and *D. novamexicana*. Following collection and arraying eggs produced by females of each species, high hatch rates were observed for both species when mated with males of the same line. Hatch rate measured for eggs produced by *D. novamexicana* is 97.2% while hatch rate measured for eggs produced by *D. americana* is 89.7%

Table 1: Progeny numbers and egg hatch rate for crosses within and between *D. americana* (NN97.4-red) and *D. novamexicana* (1031.4).

| Female parent | Number of progeny (±S.E) | | Egg hatch rate (±S.E.) | |
|---|---|---|---|---|
| | Conspecific | Heterospecific | Conspecific | Heterospecific |
| *D. americana* | 73.5 (±0.87) | 23.4 (±0.33) | 0.90 (±0.009) | 0.36 (±0.03) |
| *D. novamexicana* | 69.2 (±0.44) | 1.4 (±0.08) | 0.97 (±0.009) | 0.008 (±0.003) |

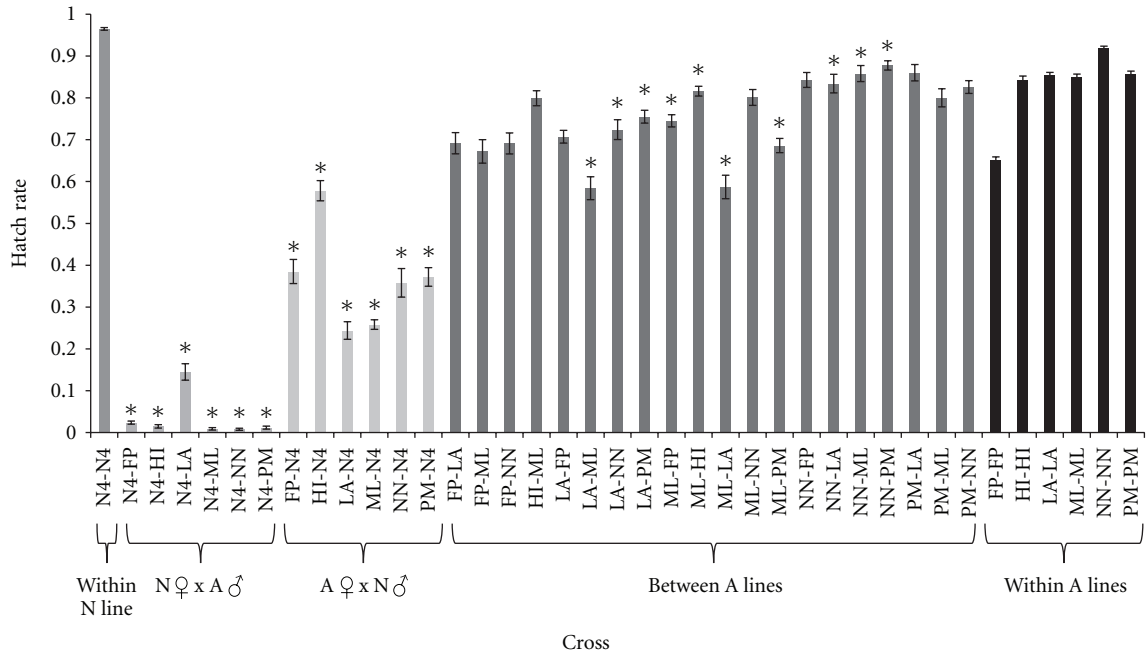
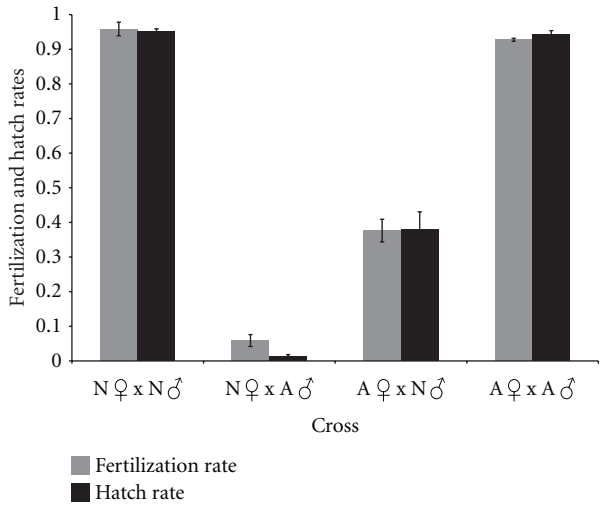



Figure 1: Egg hatch rate within and between *D. novamexicana* and six lines of *D. americana*(see Section 2). Egg hatch estimates "within N line" and "within A lines" are averages of within-line hatch rates from all experiments. Asterisks indicate egg hatch estimates that are significantly different between lines compared to within lines ($P < 0.001$, Tukey-Kramer HSD). A = *D. americana*, N = *D. novamexicana*. N4 is the *D. novamexicana* line used throughout the study (1031.4). All remaining two-letter abbreviations are the first two letters of each *D. americana* iso-female line i.d. (see Section 2).

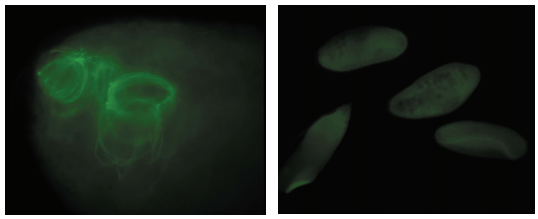
(Table 1). Hatch rates for eggs produced from heterospecific crosses are reduced, consistent with the pattern observed for progeny numbers. Only ~1% of eggs produced by female *D. novamexicana* mated with *D. americana* hatch successfully (Table 1), reflecting a significant reduction in comparison with eggs produced by females mated with conspecific males ($t = -105.6$, $d.f. = 18$, $P < 0.001$). Hatch rate of eggs produced by female *D. americana* mated with *D. novamexicana* is 35.8% (Table 1), which is significantly reduced in comparison with the conspecific cross ($t = 15.34$, $d.f. = 18$, $P < 0.001$).

Crosses within and between multiple lines of *D. americana* reveal considerable variation in hatch rate, ranging from 65.07% to 91.85% for crosses within lines and from 58.4% to 87.75% for crosses between lines (Figure 1). Overall, the average hatch rate for eggs produced from matings within lines is not significantly different from the average hatch rate from matings between lines (Tukey-Kramer HSD, $P > 0.05$), although significantly reduced hatch rates were measured in crosses between several lines of *D. americana* compared to within-line controls (Figure 1). Unlike *D. americana*, hatch rate estimates between three iso-female lines of *D. novamexicana* are uniformly high with no significant difference from within-line hatch rates (data not shown).

Hatch rates were consistently reduced for eggs produced from interspecific crosses (Figure 1). Eggs of female *D. novamexicana* crossed with males from five different lines of *D. americana* exhibit the lowest hatch rates, ranging from 0.9% to 14.5%, with each significantly lower than the conspecific hatch rate within *D. novamexicana* in each respective experiment (Tukey-Kramer HSD, $P < 0.001$). In reciprocal crosses between females of the five lines of *D. americana* and male *D. novamexicana*, hatch rates range from 24.4% to 57.8%, which is significantly reduced in comparison to hatch rates within lines of *D. americana* (Tukey-Kramer HSD, $P < 0.001$). Hatch rates in the reciprocal crosses between *D. novamexicana* and *D. americana* reflect the observations from progeny numbers; reductions in egg hatch and adult progeny are greatest in heterospecific crosses involving female *D. novamexicana*, and less severe in heterospecific crosses involving female *D. americana*.




(a) Proportion of fertilized and hatched eggs



(b) Fertilized (left) and unfertilized eggs (right)

Figure 2: (a) Proportion of fertilized and hatched eggs (error bars represent standard error). (b) Egg containing fluorescently labeled sperm tail (left) and eggs containing no sperm tail (right).

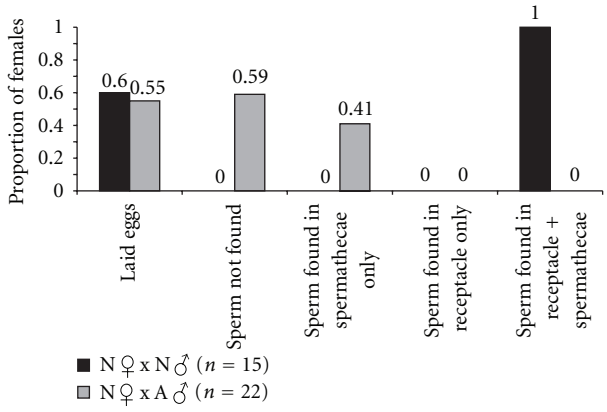
*3.3. Reproductive Incompatibility.* Reduced egg hatch in heterospecific crosses may be due to postcopulatory problems arising before fertilization, during fertilization, or early in embryogenesis. A sperm-tail specific antibody, $\alpha$-XT, was used to visualize sperm tail in laid eggs to determine whether they were successfully fertilized. Eggs were classified as fertilized if the sperm tail was clearly visible in the anterior part of the egg (Figure 2(b)). No case of partial or incomplete fertilization was observed as has been previously described in crosses between two races of *D. melanogaster* [32]. The results, shown in Figure 2(a) alongside hatch rate measurements from eggs produced from the same set of crosses, indicate a direct correspondence between the proportion of fertilized eggs and the proportion of hatched eggs in all crosses. Although the hatch rate of eggs laid by *D. novamexicana* females mated to *D. americana* males is slightly higher than the fertilization rate, indicating possible additional incompatibilities early in development, this difference is not significant ($z = 0.397$, $d.f. = 1$, $P > 0.05$). No difference is observed between hatch rate and fertilization rate in the other three crosses ($z \approx 0$, $d.f. = 1$, $P > 0.05$). This result indicates that the failure of heterospecific sperm to fertilize is the main, if not the only, contributor to reduced hybrid production and suggests minimal postzygotic incompatibility in early embryogenesis.

The reduced capacity for heterospecific sperm to successfully fertilize may arise during storage in the female reproductive tract, or may reflect the heterospecific sperm's inability to penetrate the egg. To assess the former, we investigated sperm motility and storage dynamics in both conspecific and heterospecific inseminations under two experimental regimes: one in which females were provided with standard cornmeal food and yeast immediately after insemination, and another in which females were provided with only a water source (2% agar) immediately after insemination. To maximize detection of a difference in these experiments, only crosses involving *D. novamexicana* females were performed.

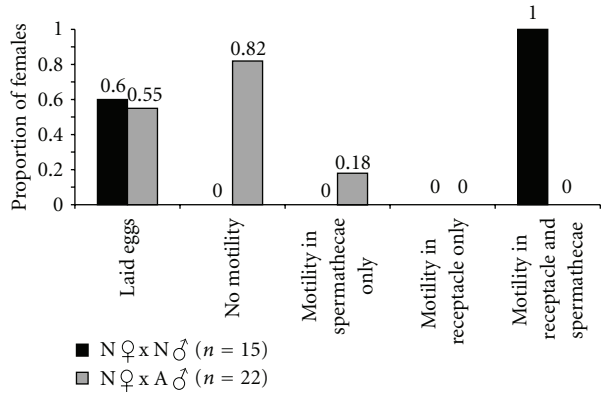
In the first regime, females were dissected at four consecutive intervals after insemination (6 hrs, 24 hrs, 48 hrs, and 72 hrs after insemination). The reproductive tracts from dissected females at each time interval were observed under phase microscopy, where motile sperm appear as colored lines. This method does not provide a quantitative measurement of sperm motility, but rather a qualitative assessment of whether sperm, if present, are motile or not. The pattern of sperm motility within storage organs was indistinguishable between conspecific and heterospecific inseminations up to three days after insemination (results not shown).

To further investigate the dynamics of sperm storage and motility within the female reproductive tract, we employed a strategy in which we kept inseminated females in suboptimal ovipositing media, namely, 2% agar. The goal here is to reduce sperm utilization by reducing oviposition propensity and to assess whether a difference in storage and motility is detectable between conspecific and heterospecific inseminations following a prolonged period of storage. To validate that egg-laying was reduced by maintaining inseminated females on 2% agar, we divided the two classes of inseminated females (conspecific and heterospecific) into two rearing conditions after insemination: a subset of each class was placed in standard cornmeal, yeasted media, and another subset on 2% agar (1 female/vial). We monitored the number of eggs laid in each vial; all inseminated females reared in standard media laid a large number of eggs, whereas half of all inseminated females reared on 2% agar laid eggs, 31 being the highest number of eggs laid, which is less than half of what would be conservatively expected under optimum conditions ($\approx$71 eggs/insemination, calculated from Table 1). This suggests that rearing inseminated females on 2% agar reduces oviposition, and potentially prolongs sperm storage.

For each inseminated female reared for 7 days on 2% agar, the intact ventral receptacle and the two spermathecae were dissected and separately classified according to whether they contained sperm, and whether the sperm was motile. The results are divided into Figures 3(a) and 3(b) representing sperm storage and sperm motility, respectively. In conspecific inseminations, sperm was found in all cases to be stored in both storage organs, whereas 41% of heterospecifically inseminated females contained sperm only in the spermathecae (Figure 3(a)) ($\chi^2 = 13.66$, $d.f. = 1$, $P < 0.001$). Figure 3(b) shows the motility status of the stored sperm: in conspecific inseminations, motile sperm was detected in both storage organs in all inseminated females, whereas only 18% of heterospecifically inseminated

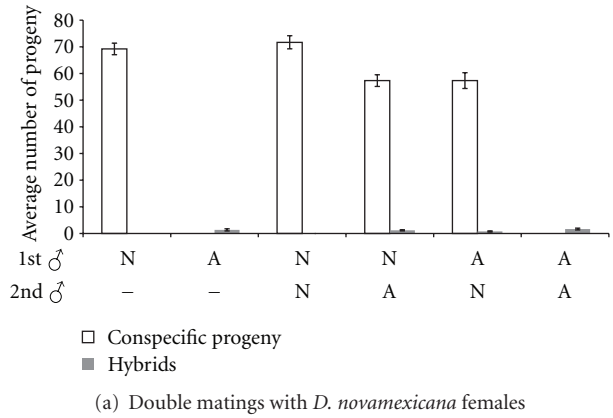



(a) Sperm storage

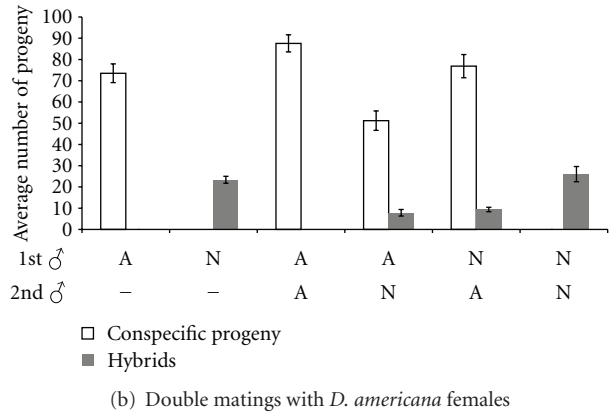



(b) Sperm Motility

Figure 3: Proportion of inseminated females reared in 2% agar that contained (a) stored sperm and (b) had motile sperm. The left-most column in both (a) and (b) shows the proportion of conspecifically and heterospecifically mated females that laid eggs in 2% agar.




(a) Double matings with *D. novamexicana* females




(b) Double matings with *D. americana* females

Figure 4: Average number of progeny produced by (a) *D. novamexicana* and (b) *D. americana* females when singly and doubly mated to conspecific and heterospecific males (error bars represent standard error).

females contained motile sperm, which was found only in their spermathecae ($\chi^2 = 23.90$, $d.f. = 1$, $P < 0.001$). These results clearly indicate that there is substantial loss of sperm in the heterospecific cross, particularly from the seminal receptacle. The low percentage of motile sperm that is found only in the spermathecae suggests that sperm may be further incapacitated or rendered inviable after prolonged storage in this organ.

*3.4. Progeny Numbers in Double Matings.* Double mating experiments were designed to examine two postcopulatory phenomena: (1) whether the ejaculate of heterospecific males incapacitates the female reproductive tract or otherwise directly reduces the reproductive success of conspecific sperm and (2) whether the presence of the conspecific ejaculate influences the reproductive success of heterospecific sperm. The results for single and double matings are summarized in Figure 4.

First, a comparison between conspecific crosses involving a single male and double matings involving a heterospecific male reveals a modest, yet significant reduction in conspecific progeny numbers produced by female *D. novamexicana* mated with males of both species irrespective of mating order (Figure 4(a); N-A: $t = 4.26$, $d.f. = 48$, $P < 0.05$. A-N: $t = 4.12$, $d.f. = 45$, $P < 0.05$.). The consistent reduction in conspecific progeny suggests that the *D. americana* ejaculate partially incapacitates the female *D. novamexicana* reproductive tract and/or directly interferes with conspecific sperm. Conversely, fewer conspecific progeny are produced in double matings involving female *D. americana* only when *D. novamexicana* is the second copulating male ($t = 4.11$, $d.f. = 37$, $P < 0.05$), whereas conspecific progeny numbers are not reduced when *D. novamexicana* copulates first (Figure 1(b): $t = 0.61$, $d.f. = 36$, $P > 0.05$). The ejaculate of *D. novamexicana* either interferes with or displaces resident conspecific sperm in the female reproductive tract of *D. americana*, but the female reproductive tract is not incapacitated since similar progeny numbers are produced from single matings with male *D. americana* and from double matings where *D. novamexicana* copulates first.

Second, we investigated whether conspecific sperm precedence (CSP) is operating in double inseminations. The main indication of CSP is a reduction in the number of hybrid progeny when a female is inseminated by a heterospecific and conspecific male relative to a female singly

inseminated by a heterospecific male. Both orders of double matings involving female *D. novamexicana* produce an equal number of hybrid progeny as the single heterospecific cross (N-A: $t = 0.47$, $d.f. = 48$, $P > 0.05$; A-N: $t = 1.67$, $d.f. = 45$, $P > 0.05$) (Figure 4(a)). However, since only one hybrid offspring is produced on average by each female *D. novamexicana* mated with male *D. americana*, CSP would be difficult to detect. A female *D. americana* on the other hand produce an appreciable number of hybrids when mated with *D. novamexicana*. When mated to both a conspecific and a heterospecific male, female *D. americana* produce fewer hybrids irrespective of mating order (A-N: $t = 8.23$, $d.f. = 37$, $P < 0.05$; N-A: $t = 7.302$, $d.f. = 36$, $P < 0.05$) (Figure 4(b)). These results indicate that the *D. americana* ejaculate reduces the fertilization success of *D. novamexicana* sperm when both are present in the reproductive tract of female *D. americana*.

## 4. Discussion

This study reveals evidence of strong postmating, prezygotic isolation between the two closely related species *D. americana* and *D. novamexicana*. This barrier to successful fertilization following insemination appears to arise in both species from an incompatibility between the female reproductive tract and the male ejaculate of the other species. Furthermore, prolonged storage of sperm in the reproductive tract of *D. novamexicana* leads to loss and/or incapacitation of heterospecific sperm. Our method of minimizing oviposition by keeping inseminated females in 2% agar allowed us to detect differences in storage dynamics and sperm motility between conspecific and heterospecific inseminations of *D. novamexicana*. Our observations of sperm motility lack quantitative measures (which are difficult to obtain); however, we were able to detect a global difference in sperm motility between conspecific and heterospecific crosses involving *D. novamexicana*. We are unable to account for a possible difference in number of sperm transferred by males from each species during copulation; however, this difference is unlikely given that conspecific crosses in both species produce a similar number of progeny. Storage and motility dysfunctions may not be the only disruptions to successful fertilization since a direct inability of sperm to penetrate the egg cannot be ruled out. It is also possible that the recognition mechanism between sperm and egg is compromised in heterospecific crosses given that sperm is successfully stored and maintains motility for at least 72 hours after insemination in optimum ovipositing conditions (results not shown). Therefore, other possible mechanisms preventing fertilization cannot be excluded.

Egg hatch estimates between *D. novamexicana* and all six lines of *D. americana* used in this study demonstrate that the postmating, prezygotic barrier is indeed a species-wide phenomenon. Crosses between three *D. novamexicana* iso-female lines show minimal variation in egg hatch rates (data not shown), perhaps reflecting the species' overall paucity of genetic variation. On the other hand, half of the crosses between different *D. americana* iso-female lines show significant hatch rate reductions when compared to within-line controls, although these reductions are not as dramatic as those observed between species. These within species reductions do not relate to the historically recognized subdivision between northern and southern forms of *D. americana*, but rather may reflect the high genetic variability maintained within this species. In conjunction with the pattern of genetic differentiation characterizing the two species (that alleles of *D. novamexicana* are mostly recovered as a relatively invariant subset of the larger variation segregating in *D. americana*), these observations hint at possible evolutionary dynamics causing rapid divergence of this gametic incompatibility (see below), and provide a few plausible explanations for the asymmetry in fertilization success in reciprocal heterospecific crosses. Consider, for instance, the copulatory environment encountered by females of each species in their respective ranges. *D. americana* females encounter diverse alleles from their conspecific males, and therefore require a reproductive tract that is permissive to a wide variety of genotypes in order to maximize their fertilization success. *D. novamexicana* females, on the other hand, encounter their invariant conspecifics, and therefore variation in their reproductive tract's permissiveness is unnecessary. This may explain why *D. americana* females utilize heterospecific sperm more efficiently than *D. novamexicana.* Alternatively, genetic drift may simply override selection in the smaller *D. novamexicana* population causing random loss of maternal alleles that may be more compatible with *D. americana* males. Higher genetic variation in *D. americana* may also contribute to stronger selective outcomes for males through postcopulatory sexual selection and/or genetic conflict, leading to higher divergence in paternal alleles of *D. americana* relative to *D. novamexicana*. One line of *D. americana*, LA99.46, is unique in that its hatch rate with *D. novamexicana* females is 14.5% compared to ~1% in all other *D. americana* lines examined. Lower incompatibility in this cross may be due to paternal alleles segregating in the *D. americana* population that are more compatible with *D. novamexicana* females, and possibly present at low frequency. Until the interacting genetic components in both males and females are known, the evolutionary genetic causes of reduced fertilization in reciprocal heterospecific crosses remain speculative.

Interspecies ejaculate competition was assessed using double mating crosses, which provided insight on whether the heterospecific ejaculate plays a role in reducing reproductive success of conspecific sperm (interference), and whether the species display conspecific sperm precedence (CSP). The former may be due to either direct interference by heterospecific sperm (e.g., competition for fertilization) or by indirectly compromising the female's reproductive tract through effects imposed by the heterospecific ejaculate. We observe interference in female *D. novamexicana* irrespective of mating order, consistent with indirect interference through females. This is not observed for female *D. americana*, where conspecific progeny number is reduced only when a conspecific copulation is followed by a heterospecific copulation, possibly due to second male precedence. Further experiments are needed to elucidate the details of this phenomenon.

Double mating with female *D. americana* reveals the presence of CSP, because the number of hybrids produced is significantly decreased in the presence of the conspecific ejaculate. This phenomenon in insects (competitive gametic isolation [1]) has been observed in ground crickets [33] and other *Drosophila* species [34, 35] for which the likely mechanism is sperm displacement [36]. A recent study of storage dynamics within *D. melanogaster* females doubly inseminated by two strains differing in fluorescently labeled sperm heads reveals that second male precedence is accomplished through displacement of resident sperm [37]. In the current study the mechanism of CSP is unknown, although we expect that it may be due to the higher competitive ability of conspecific sperm. It is also likely that higher competitive ability and second male precedence jointly contribute to reduced hybrid production in doublymated *D. americana* females when *D. americana* mates second.

Gametic isolation between species may be a result of incompatibilities that evolve as a result of male-male competition and male-female coevolution within polyandrous species [38]. Sperm competition on the one hand increases male reproductive success at the expense of female fitness, to which females in turn evolve means to counteract the deleterious ejaculate effects [39–41]. This process leads to an arms-race dynamic between males and females and results in rapid evolution of interacting reproductive systems. Coevolution between the sexes also results in rapid evolution of sex-specific phenotypes, such as sperm length [42], which has been shown to correlate with higher fertilization success. In other words, these within-species dynamics result in cryptic sexual selection that likely accelerates divergence of the genes underlying sexual interactions. Seminal fluid proteins (Acps) in *Drosophila*, which facilitate many of the postcopulatory processes leading to successful fertilization (reviewed in [43]), have been shown to evolve rapidly (e.g., [44]). Our study shows that gametic incompatibility between species, which may also result from a coevolutionary dynamic within species, has arisen much more rapidly between species than within species, suggesting a role for allopatric separation in homogenizing the coevolutionary sexual interactions within species, but deeming those interactions incompatible between species. It is therefore likely that some form of cryptic sexual selection is causing the rapid divergence of traits required for proper storage, gamete recognition, and fertilization.

*D. americana* and *D. novamexicana* represent a special case in the literature because they are the only reported *Drosophila* species pair where gametic isolation is the only apparent reproductive barrier detectable in the laboratory. Given their recent divergence and allopatric distribution, they represent a unique opportunity to study the genetics of earlystage postmating, prezygotic isolation in *Drosophila*, studies that have been lacking until recently. Crosses among all four members of the *virilis* group show gametic incompatibilities with varying degrees of asymmetry and severity [7, 8], suggesting that postmating, prezygotic isolation is common and may be particularly apparent in this phylad due to the low level of postzygotic incompatibility. Genetic analysis of this form of isolation in this species group will allow us to determine whether the evolutionary dynamics characterizing postzygotic isolation (rapid evolution by positive selection) also characterize postmating, prezygotic barriers.

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*Review Article*

# A Phenotypic Point of View of the Adaptive Radiation of Crested Newts (*Triturus cristatus* Superspecies, Caudata, Amphibia)

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The divergence in phenotype and habitat preference within the crested newt *Triturus cristatus* superspecies, examined across different ontogenetic stages, provides an excellent setting to explore the pattern of adaptive radiation. The crested newts form a well-supported monophyletic clade for which at least the full mitochondrial DNA phylogeny is resolved. Here we summarise studies that explored the variation in morphological (larval and adult body form, limb skeleton, and skull shape) and other phenotypic traits (early life history, developmental sequences, larval growth rate, and sexual dimorphism) to infer the magnitude and direction of evolutionary changes in crested newts. The phenotypic traits show a high level of concordance in the pattern of variation; there is a cline-like variation, from *T. dobrogicus*, via *T. cristatus*, *T. carnifex*, and *T. macedonicus* to the *T. karelinii* group. This pattern matches the cline of ecological preferences; *T. dobrogicus* is relatively aquatic, followed by *T. cristatus*. *T. macedonicus*, *T. carnifex*, and the *T. karelinii* group are relatively terrestrial. The observed pattern indicates that phenotypic diversification in crested newts emerged due to an evolutionary switch in ecological preferences. Furthermore, the pattern indicates that heterochronic changes, or changes in the timing and rate of development, underlie the observed phenotypic evolutionary diversification.

## 1. Introduction

Exploring patterns of phenotypic variation during ontogeny and phylogeny is fundamental to gaining insights into the processes of evolutionary diversification, including the mechanisms of speciation. The connection between development, evolutionary history, ecology, and morphology has intrigued evolutionary biologists for over the 150 years since Darwin first published his ideas about natural selection [1]. This is largely due to the idea that phenotypic evolution might be explained by changing or truncating the ancestral ontogeny, for which the characteristics can be inferred through phylogenetic analyses [2–4].

Within a monophyletic group of closely related species, it is expected that shared evolutionary history is reflected by phenotypic similarity, due to a shared developmental basis inherited from a common ancestor. Adaptive radiation and morphological divergence are usually attributed to differential selection acting upon geographical populations. In other words, "ecological opportunity" could lead to adaptive radiation [5, 6]. When phenotypic divergence is paralleled in multiple complex phenotypic traits with separate developmental pathways, this is indicative of adaptation to differential environmental selection pressures [6–9]. This line of reasoning is especially strong in situations where phenotypic variation correlates with different ecological demands.

The crested newts (*Triturus cristatus* superspecies) are an example of an adaptive radiation. Crested newts have been used as model organisms in various studies in evolutionary biology, including the processes and outcomes of speciation [10–14]. The phenotypic and ecological divergence in crested newts, examined across different ontogenetic stages, provides an excellent model to explore the tradeoff between shared evolutionary history and divergent functional requirements within an adaptive radiation.

Here we summarise data from previous studies on crested newts. The resulting framework can be used to evaluate how developmental and functional processes have impacted phenotypic evolution.

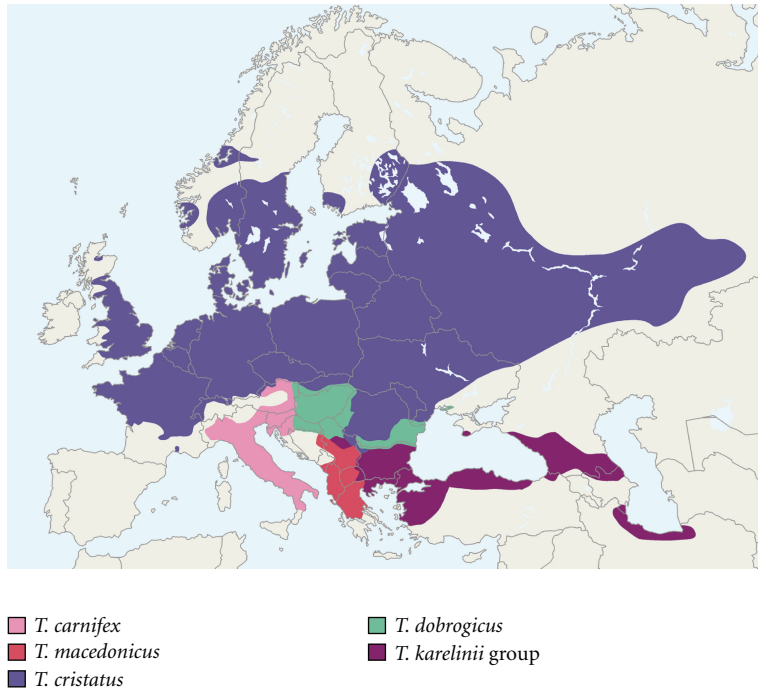


Figure 1: The approximate ranges of the crested newt species. The distribution of *Triturus carnifex*, *T. macedonicus*, *T. cristatus*, *T. dobrogicus*, and the *T. karelinii* group, illustrated after [14–17].

## 2. About Crested Newts

Crested newts belong to the group of the Modern Eurasian newts [33]. Crested newts have a biphasic life cycle with aquatic larvae that metamorphose into terrestrial juveniles and as adults return to the water annually to breed [34, 35]. Crested newts have low mobility, a small dispersal range, and strong philopatric behaviour, which promote genetic isolation. The crested newts form a well-supported monophyletic clade of closely related species for which the molecular phylogeny has been largely resolved [11, 14, 33, 36]. According to current taxonomy, this group consists of six species: *T. cristatus*, *T. dobrogicus*, the closely related species *T. carnifex* and *T. macedonicus* [11], and two species that belong to the so-called *T. karelinii* group, *T. karelinii* and *T. arntzeni*. Mitochondrial DNA studies showing three distinct clades within the *T. karelinii* group [14, 15] illustrate that the taxonomy of the *T. karelinii* clade is as yet unsettled. In an attempt to simplify, we will refer to these newts as the *T. karelinii* group.

The range of the crested newts spans most of Europe and adjacent Asia (Figure 1). The nominotypical species (*T. cristatus*) is the most widely distributed over much of Europe. *T. dobrogicus* is confined to the Pannonian area and the Danube Delta and Dobrugea Plain. The other species have a more southern distribution, restricted to the Apennine Peninsula and the northeast Balkan Peninsula (*T. carnifex*), the western Balkan Peninsula (*T. macedonicus*) and the eastern Balkan Peninsula, Asia Minor, Crimea, Caucasus, and southern shore of the Caspian Sea (*T. karelinii* group). The species have a parapatric distribution and a potential to interbreed along the contact zones, especially in the Balkan region [37, 38].

The crested newt species differ in their ecological demands [18, 19]. The most aquatic is *T. dobrogicus*, followed by *T. cristatus*. Generally, *T. macedonicus*, *T. carnifex* and the *T. karelinii* group are the more terrestrial species. *T. dobrogicus* inhabits permanent and/or long-lasting, large, stagnant water bodies. *T. cristatus* occupies mostly long-lasting medium-sized water bodies. The other crested newt species are associated with relatively small lentic ponds with variable hydroperiods [18, 37]. The duration of the aquatic phase is directly related to their habitat and varies from a short, three months in the *T. karelinii* group up to six months in *T. dobrogicus* (Figure 2). It is worth to note that the evolution of habitat preferences of crested newts still needs to be addressed properly.

The most notable characteristic of the crested newts' origin is that their evolutionary splitting occurred within a short-time span, which indicates that there was a burst of speciation rather than a prolonged process of speciation [11, 14, 36]. This is typical for adaptive radiations. Ecologically based, spatially heterogeneous selection, coupled with limited migration, can result in rapid phenotypic diversification [39, 40]. Such a scenario presumes the existence of divergent ecological conditions, as well as a low magnitude of phenotypic and genetic correlations. A high level of phenotypic divergence could be achieved even under substantial hybridisation and gene flow [41].

## 3. Interspecific Variation in Phenotypic Traits

*3.1. Adult Body Form.* The term "body form" refers to the robust morphological features of an organism's external morphology and encompasses both size- and shape-related

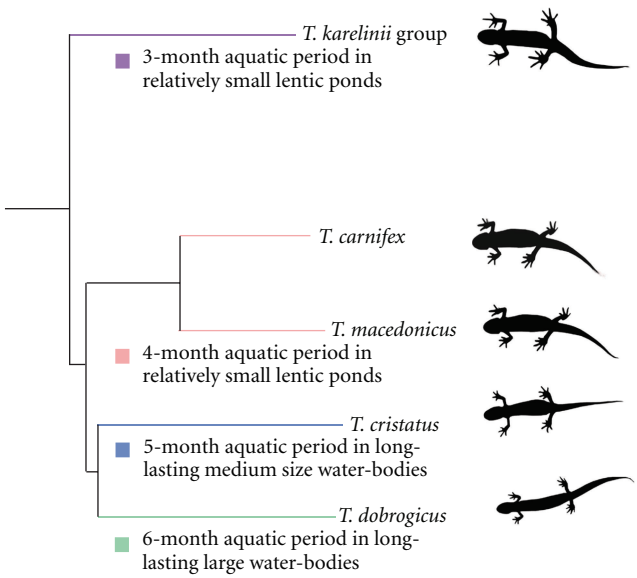


Figure 2: The phylogeny, the habitat preferences and the ecological demands of the crested newt species [18, 19]. To infer the direction of evolutionary changes among crested newt species the latest, most complete phylogenetic analysis [14] was used. The branch lengths are proportional to the number of base substitutions per site [14].

characteristics. Body form can differ between species, but also between groups of species. In tailed amphibians, adaptation to an aquatic life is usually related to body elongation and limb reduction, which may increase swimming performance. A compact body and robust limbs are linked to a terrestrial life and a lack of passive buoyancy.

Crested newts show a range body forms from a slender body and short limbs in *T. dobrogicus,* via *T. cristatus,* and *T. macedonicus* and *T. carnifex* to a short body and long limbs in the *T. karelinii* group [37, 42]. The ancestral phenotype, a large body with a short trunk and a wide head, characterises the *T. karelinii* group. The species *T. carnifex* and *T. macedonicus* have a large body and wide head accompanied by mild body elongation. The most derived phenotype includes body size reduction and more pronounced body elongation in *T. cristatus* and, especially, in *T. dobrogicus* (Figure 2). Body elongation in these newts is reflected in the modal number of rib-bearing vertebrae: 13 in *T. karelinii* group, 14 in *T. macedonicus* and *T. carnifex*, 15 for *T. cristatus,* and 16 or 17 in the most elongated *T. dobrogicus* [14, 37, 43, 44].

*3.2. Ontogeny of Body Shape.* The ontogenetic niche shift and transition between the aquatic and terrestrial habitats is coupled with metamorphosis and an overall change in the relationship between the individual and its environment. Therefore, two different sets of adaptations and constraints during growth could shape the ontogenetic trajectories of crested newts and affect their phenotypic diversification. The analysis of ontogenetic shape changes and changes in developmental rate [20] gives insight into the processes of the evolutionary diversification of the crested newts. The four analysed species of crested newts (*T. dobrogicus, T. cristatus, T. macedonicus,* and *T. arntzeni* (*T. karelinii* group), differ in size and shape as larvae, at least when the larval body form is fully developed (i.e., midlarval stage). The ontogenetic trajectories of larval shape diverge in both the direction and the rate of shape changes along species-specific trajectories [20]. The species significantly differ in their developmental rate of larval shape, except for *T. cristatus* and *T. dobrogicus*, which are similar. *T. dobrogicus* clearly differed from the other species in having a higher and wider caudal fin, while *T. arntzeni* (*T. karelinii* group) has the most elongated larvae with the lowest tail fin (Figure 3). Based on the assumption that the shape of caudal fins is of high adaptive significance for larvae, it is tempting to hypothesise that the two larval shape types represent aquatic ecological adaptations of the two species groups of crested newt.

Contrary to clear discrimination between species in larval body shape, at the juvenile stage just after metamorphosis, the species converge on a similar body shape [20]. After that stage, crested newt species apparently diverge toward the adult body shape. The differences in body shape that we found may indicate that the body forms of larvae and adult individuals are subject to selection in both the aquatic and terrestrial environments, resulting in the same pattern of interspecific differences, despite the possibility of two distinct sets of constraints [45].

*3.3. Limb Size, Ossification Level, and the Pattern of Morphological Integration.* A morphometric analysis of the limb skeleton of four crested newt species (*T. dobrogicus, T. cristatus, T. carnifex,* and *T. arntzeni* (*T. karelinii* group)) showed that although they differ in the size of skeletal elements (stylopodium, zeugopodium, and third metapodial element), they all shared common allometric slopes [30]. A similar relationship between limb skeleton size and body size could indicate a conservative direction of ontogeny [30]. However, a lateral shift in the species-specific allometries of *T. dobrogicus* and *T. cristatus* indicates evolutionary changes in the allometric trajectories. The lateral shift could indicate that heterochronic changes underlie the observed morphological variation [3]. Moreover, the *T. dobrogicus* manus has a significantly lower ossification level and concomitant loose "bone packaging" compared with the other species (Figure 4), which could be a result of the heterochronic changes [46, 47].

Limbs, as serially homologous structures, share a strong developmental basis. The shared genetic factors (e.g., *Hox* patterning genes) are intrinsic to the covariation among the homologous structures within limbs (e.g., radius and tibia or humerus and femur) and the overall morphological integration [48, 49]. Epigenetic factors, such as function, also could have an impact on limb integration. In empirical studies, the expression of functional and developmental interdependencies in the patterns of integration could be estimated. If covariation between the homologous parts of the fore- and hindlimbs is stronger than the covariation of skeletal elements within the limbs, then developmental constraints prevail over functional determinants [49–51]. Two opposing correlation patterns were observed [13] in the more terrestrial species, the homologous limb elements were less correlated, and the within-limb elements were more correlated, whereas, in the aquatic species, the reverse pattern

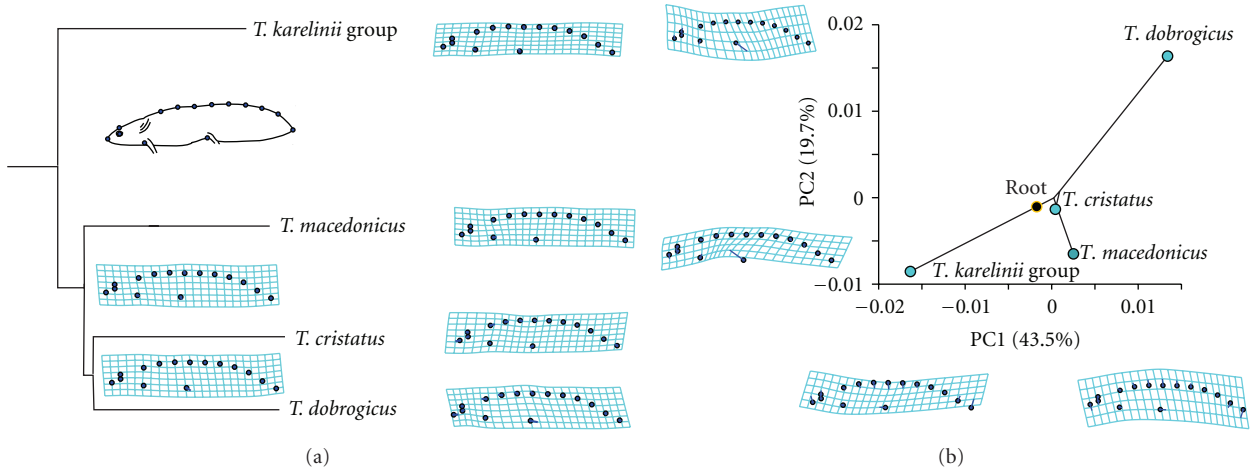


Figure 3: The shape changes of 90-day-old larvae mapped on the crested newt phylogeny (a) and the phylogeny superimposed in the morphospace defined by the first two principal axes (b). To capture larval shape, both landmarks and semilandmarks presenting the shape of dorsal caudal fin at larvae and tail shape were used [20]. We applied a procedure for mapping the geometric morphometric data onto a known phylogeny [21–23]. The criteria of squared-change parsimony (weighted by divergence time or molecular change on the respective branches of the tree) were used for reconstructing the values of the internal nodes of the phylogeny from the shape averages of the terminal taxa [24–27]. We used the generalised method of least squares [26, 28] to find values for the internal nodes. The sum of squared changes along the branches is minimised over the entire phylogeny. We applied evolutionary principal component analysis [21], and the ordination of mean shapes in the space of the first two principal axes is presented. The thin-plate spline deformation grids that illustrate lateral larval shape changes correlated with the first and the second axis are presented [22]. The analyses and the visualisation of shape changes in the evolutionary morphospace were performed using the MorphoJ software [29].

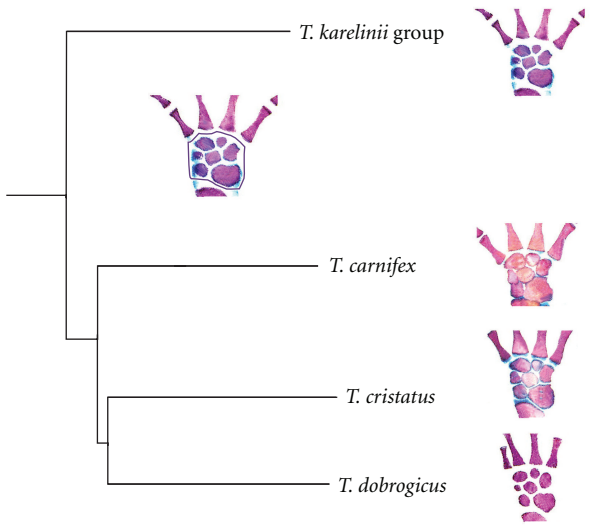


Figure 4: The forelimbs of four crested newt species that illustrate differences in "bone-packaging" [30]. Bones are coloured in red, cartilage in blue, and the surrounding soft tissue is clear and semitransparent. The statistically lower ossification level and concomitant loose "bone packaging" characterise *T. dobrogicus*.

occurred (Figure 5). All of these results indicate that function appears to be the covariance-generating factor that has shaped the patterns of morphological integration of crested newt limbs.

*3.4. Skull Shape and Ontogenetic Skull Shape Changes.* Crested newts differ in skull shape [32]. The visualisation of the phylogeny superimposed in the morphospace, and the positions of the internal nodes in the phylomorphospace (Figure 6) indicate that most of the shape changes occurred along species-specific branches. *T. dobrogicus* markedly diverged in skull shape as reflected by a more slender and elongated skull (Figure 6). The advantages of such morphology might be better locomotion in aquatic habitats due to a more streamline body shape but with the possible disadvantage of reduced abilities of suction feeding. The similarities in skull shape of *T. macedonicus* and *T. arntzeni* (*T. karelinii* group) probably reflect a symplesiomorphy.

The analysis of the ontogenetic trajectories of skull shape changes between juveniles just after metamorphosis and adults [12] indicate that *T. dobrogicus* has the highest rate of cranial shape change during postmetamorphic growth, as well as a distinctive ontogenetic allometric trajectory compared with the other three analysed species (*T cristatus*, *T. carnifex*, and *T. arntzeni* (*T. karelinii* group)). To visualise the direction and amount of shape changes during crested newt skull shape ontogeny (Figure 7), we performed an additional analysis using a larger sample of juveniles than available for the study of ontogenetic shape changes [12]. The slope and the amount of ontogenetic shape change of *T. dobrogicus* clearly diverged from the other species. It is interesting to note that the changes in skull shape that clearly separate *T. dobrogicus* and *T. cristatus* from *T. macedonicus* and the *T. karelinii* group are shared in both analysed stages: the juvenile stage just after metamorphosis and the adult stage (Figure 7).

*3.5. Early Ontogeny.* Comparative study of crested newt development and early life history traits, such as egg characteristics, developmental rate, survival rate, and duration of

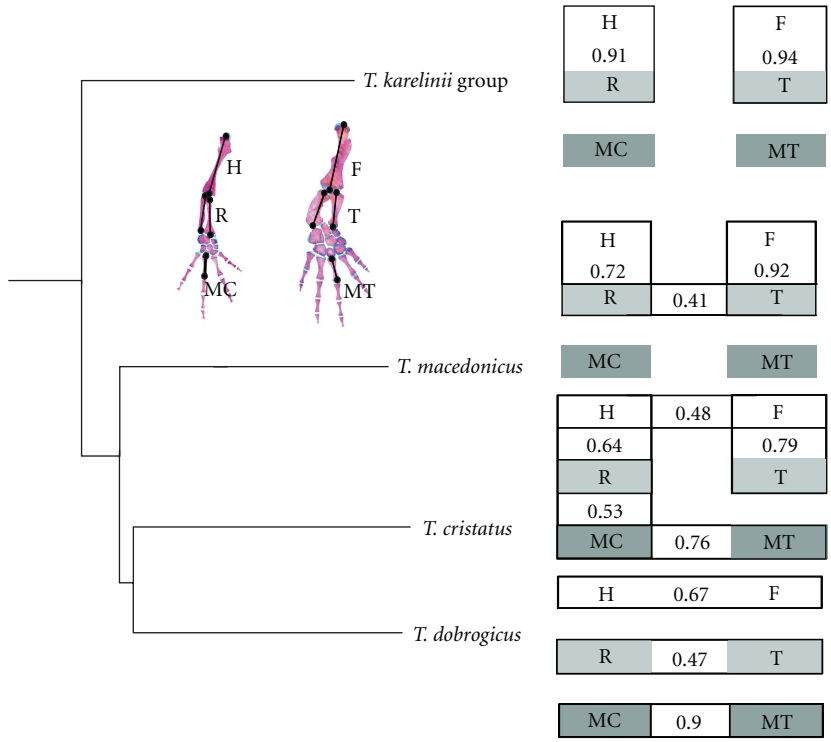


Figure 5: Graphical model of the significant partial correlations illustrated as boxes between limb elements ($P < 0.05$) of adult individuals [13]. H: humerus; R: radius; MC: metacarpal; F: femur; T: tibia; MT: metatarsal. Partial correlations measure a correlation between two variables that are independent of information from the other variables in the correlation matrix. The significance of partial correlations was assessed using an information theoretic measure known as the edge exclusion deviance (EED) and the $\chi^2$ distribution: EED $= -N \ln(1 - \rho^2_{ij\{K\}})$, where $N$ is the sample size, and $\rho^2{}_{ij\{K\}}$ is the partial correlation coefficient between variables $i$ and $j$ [31]. The two variables were conditionally independent when the EED value was less than 3.84 (corresponding to $P = 0.05$, df = 1 from the $\chi^2$ distribution). In *T. dobrogicus*, significant edges were present only between homologous limb elements. This finding is in opposition to *T. arntzeni* (*T. karelinii* group) which had high partial correlations within limbs between the stylopod and zeugopod elements. *T. arntzeni* (*T. karelinii* group) had a stronger correlation of skeletal elements within the limbs than between fore- and hindlimbs. The partial correlations between limb elements in *T. cristatus* and *T. macedonicus* were intermediate with regard to the previous species, with variable significant edges between homologous and within-limb elements.

the embryonic period [52, 53] produced data valuable for understanding the forces shaping adaptation and evolutionary diversification. In vertebrates, vitellus size appears to be one of the key life-history traits reflecting maternal input and could affect the development rate and the size and stage of larvae at hatching [54]. The vitellus size and thickness of the mucoid capsule that protects from injury, fungal infestation, and ultraviolet-B radiation were investigated in four crested newt species (*T. macedonicus*, *T. cristatus*, *T. dobrogicus,* and *T. arntzeni* (*T. karelinii* group)) [52]. Larger crested newt females tend to produce eggs with larger vitelluses. Although the studied species shared a common allometric slope of the egg size versus body size relationship, the species differed in the egg size, which appeared to be a species-specific life-history trait with a cline-like distribution; *T. dobrogicus* has the smallest eggs, the members of the *T. karelinii* group and *T. macedonicus* have the largest eggs, and *T. cristatus* has intermediate-sized eggs.

The crested newts are similar with respect to basic developmental traits (no differences in developmental sequences and survival rates). However, there is a significant variation in the developmental rate. Generally, the developmental rate highly depends on environmental factors, especially temperature. Under experimental conditions [53], *T. dobrogicus* appears to be the outlier species, particularly in comparison to *T. arntzeni* (*T. karelinii* group) and *T. macedonicus*, which have the longest developmental period [53]. Also, there are differences in the pattern of correlation amongst life-history and developmental traits. The comparisons of phenotypic correlation matrices based on eleven life history and developmental traits [53] revealed that *T. dobrogicus* have a correlation pattern similar to *T. cristatus* and *T. macedonicus*. *T. arntzeni* (*T. karelinii* group) has a similar correlation pattern to *T. macedonicus*, but there are no similarities in the matrix correlation pattern compared to the other two species.

## 4. Adaptive Radiation Pattern

Adaptive radiation refers to the evolution of ecological and phenotypic diversity within multiple lineages [41]. There are four criteria to detect an adaptive radiation [40]; common ancestry, rapid radiation, environmental correlation, and trait utility. In crested newts, common ancestry and rapid

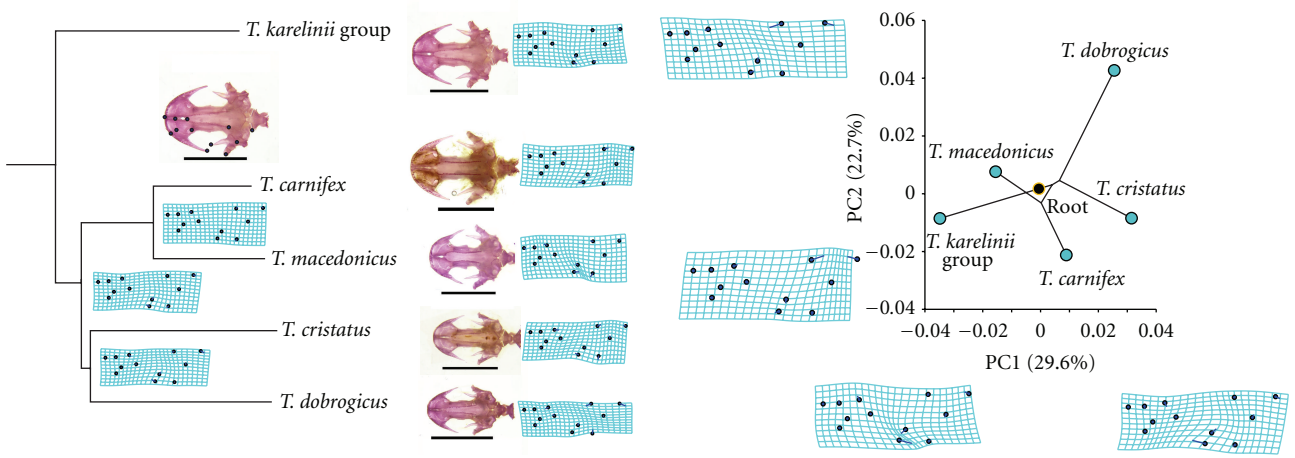


Figure 6: Skull shape changes mapped on the crested newt phylogeny (a), and the phylogeny superimposed in the morphospace defined by the first two principal axes (b). To calculate mean shape, we used a subset consisting of male specimens from population samples used for the study of variation in crested newt skull shape [32]. To visualise the changes of ventral skull shape along the crested newt phylogeny, we applied a procedure for mapping geometric morphometric data onto a known phylogeny [23]. The criteria of squared-change parsimony (weighted by divergence time or molecular change on the respective branches of the tree) were used for reconstructing the values of the internal nodes of the phylogeny from the shape averages of the terminal taxa [24–27]. We used the generalised method of least squares [26, 28] to find values for the internal nodes. The sum of squared changes along the branches is minimised over the entire phylogeny. We applied evolutionary principal component analysis [21], and the ordination of the mean shapes of five *Triturus* species in the space of the first two principal axes is presented. The thin-plate spline deformation grids that illustrate skull shape changes correlated with the first and the second axis are presented [22]. The analyses were performed using MorphJ software [29].

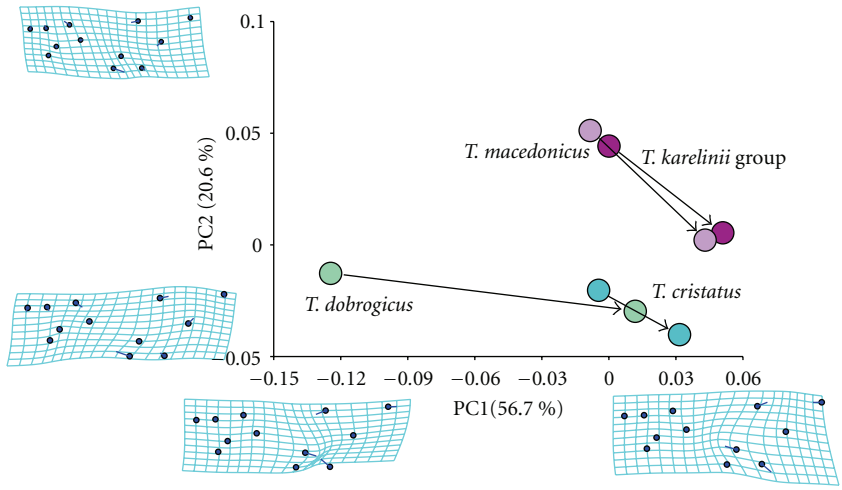


Figure 7: Postmetamorphic skull shape changes. The analysed sample of juveniles at the stage just after metamorphosis was obtained from a laboratory breeding experiment [53]. The sample of juveniles consisted of 10 specimens of *T. dobrogicus*, *T. macedonicus,* and *T. arntzeni* (*T. karelinii* group) and 5 specimens of *T. cristatus*. A general procrustes analysis [55–57] was performed for the entire sample of juvenile and adult specimens of the four analysed species. We calculated the mean shape for each stage and species and performed exploratory, principal component analysis. The positions of species- and stage-specific mean shapes in the morphospace defined by the first two principal axes are given. An arrow connecting the means for juveniles and those for adult specimens in the morphospace defined by the first two principal axes visualises the direction and the amount of shape changes during skull ontogeny.

radiation can be readily inferred from phylogenetic analysis. Also, there is a perfect match between the patterns of interspecific differentiation in phenotypic traits and ecological preferences. However, no clear relationship between evolution of body form and locomotor function in newts was found [10]. The onset of adaptive radiation often requires, in addition to the existence of a new habitat, the possession of a key innovation that allows rapid adaptation in novel ecological settings [58].

The observed pattern of differences in the crested newts' phenotypic characteristics shows that *T. dobrogicus* is in any respect the most derived species. *T. dobrogicus* has (1) the

most elongated body and the largest number of rib-bearing vertebrae [14, 42]; (2) a significantly different size, ossification level, and pattern of morphological integration of limbs [13, 30]; (3) a marked difference in skull shape [32], including the direction and rate of ontogenetic shape changes [12]; (4) the smallest vitellus [52]; (5) peculiarities in life history traits [53]; (6) a distinct pattern of sexual dimorphism of morphometric traits (the sexual dimorphism in body size is absent in *T. dobrogicus*, while in other species females are the larger sex [59]). However, from this long list of diverged phenotypic traits that characterise *T. dobrogicus*, a key innovation cannot yet be clearly recognised.

## 5. Possible Mechanism of the Crested Newts' Evolutionary Diversification in Phenotypic Traits

External restrictions imposed by ecology had a strong influence on the crested newts' phenotypic divergences, including development. In our view, the evolutionary diversification of phenotypic traits in crested newts was driven by ecological speciation. (An additional separation between crested newts might occur through parapatric speciation, in which populations diverge with some gene flow [60].)

The pattern of divergence in developmental rate and correlation pattern between several early life-history and developmental traits [52, 53] indicates that crested newt evolution seemed to be accompanied by a significant ecological diversification and by labile development patterning, including differences in developmental timing. Heterochrony, differences in the sequence of developmental events, the timing and the rates of development, are often invoked as causes that underlie observed phenotypic evolutionary changes [61]. Crested newts are prone to heterochronic changes [62]. The data collected so far suggest that heterochronic changes in early ontogeny can lead to the lateral transposition of the *T. dobrogicus* ontogenetic trajectories, as previously suggested for the cranial shape [12] and allometric limb skeleton trajectories [30].

## 6. The Ecological Shift Drives the Evolution of Phenotypic Traits in the Crested Newts

Natural selection related to shifts in ecology (e.g., invasion of new habitats) can lead to extremely rapid divergence [63]. The new, colonising populations are particularly likely to diverge, especially because they are usually small and likely to be genetically altered. Recently, it has been proposed that new species generally emerge from single events (e.g., changes in environments), and that ecological adaptation promotes reproductive isolation and speciation [58, 64, 65]. Also, a growing body of research demonstrates a link between rapid ecological divergence and speciation [66].

We advocate the hypothesis that the phenotypic diversification in crested newts emerged due to an evolutionary switch in ecological preferences [53]. The phenotypic characteristics of crested newts could have evolved over a short-time span during which the main crested newts phylogenetic lineages diverged in the central Balkans [11, 43]. The main ecological shift of *T. dobrogicus* (and less apparently of *T. cristatus*) could happen due to the availability of aquatic habitats on the Central Balkans [67, 68]. The subsequent phenotypic evolution of *T. dobrogicus* could be amplified through ecological selection, when the species occupied extensive lowland floodplains along the present-day Danube River and its tributaries which were covered with swamps and marshes. This event dated back to Pliocene when the Pannonian Sea dried out, more than three million years after *T. dobrogicus* had separated from other crested newts' species [14]. Similar changes might have occurred when *T. cristatus* spread across the European plains after Pleistocene glaciations. If so, adaptive phenotypic radiation in crested newts appears as an extension of the initial process of speciation.

For both *T. dobrogicus* and *T. cristatus,* the acquiring of new habitats by phenotypic diversification was not accompanied by additional lineage differentiation. Most likely, the high connectivity of large lowland water bodies, distributed in areas affected by glaciations, prevented the long-term partitioning of populations and thus inhibited speciation [69, 70]. In contrast, within the *T. karelinii* group, subdivision across a heterogeneous landscape, especially those in glacial refuges, promotes geographical isolation and thus speciation [15, 69].

## 7. Conclusions

The morphologically and ecologically diversified species of crested newts exhibit a cline-like variation pattern in phenotypic traits, with *T. dobrogicus* and the *T. karelinii* group on the opposite poles and *T. cristatus* as an intermediate species, while *T. carnifex* and *T. macedonicus* are close to the *T. karelinii* group. This pattern matches the cline of the species' ecological preferences indicating that phenotypic diversification in crested newts emerged most likely due to an evolutionary switch in ecological preferences. The patterns of variation also indicate that heterochronic changes underlie the observed phenotypic evolutionary changes.

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*Research Article*

# Inter- and Intraspecific Variation in *Drosophila* Genes with Sex-Biased Expression

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Genes with sexually dimorphic expression (sex-biased genes) often evolve rapidly and are thought to make an important contribution to reproductive isolation between species. We examined the molecular evolution of sex-biased genes in *Drosophila melanogaster* and *D. ananassae*, which represent two independent lineages within the *melanogaster* group. We find that strong purifying selection limits protein sequence variation within species, but that a considerable fraction of divergence between species can be attributed to positive selection. In *D. melanogaster*, the proportion of adaptive substitutions between species is greatest for male-biased genes and is especially high for those on the X chromosome. In contrast, male-biased genes do not show unusually high variation within or between populations. A similar pattern is seen at the level of gene expression, where sex-biased genes show high expression divergence between species, but low divergence between populations. In *D. ananassae*, there is no increased rate of adaptation of male-biased genes, suggesting that the type or strength of selection acting on sex-biased genes differs between lineages.

## 1. Introduction

In sexually reproducing species, the evolution of reproductive isolation is closely coupled to the process of speciation. Indeed, the widely applied biological species concept defines species as "groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups" [1]. This definition has been of great utility to geneticists working with organisms like *Drosophila* that are separated into populations or species showing varying degrees of pre- and postzygotic reproductive isolation. The degree of isolation increases with the time since the species shared a common ancestor [2, 3].

Within species, prezygotic isolation is often observed as preferential mating of individuals (usually females) to other individuals from the same population. Such behavioral isolation has been observed for *Drosophila melanogaster* and *D. ananassae* populations that diverged within the past 15,000–20,000 thousand years [4, 5]. At the postzygotic level, it is often found that matings between closely related species produce hybrid offspring in which at least one sex (usually males) is either inviable or infertile. For example, species of the *D. simulans* complex, which diverged around 0.5–1.0 million years ago [6], produce viable hybrid offspring with only the males being infertile [7, 8]. Crosses between *D. melanogaster* and *D. simulans*, which diverged around 4-5 million years ago [6], produce viable, but sterile, offspring of only one sex (the sex of the *D. melanogaster* parent) [9, 10].

The observations from *Drosophila* suggest that the evolution of postzygotic reproductive isolation is a progressive process that involves the accumulation of incompatible alleles at many loci across the genome [11–13]. Since the first stage of isolation is typically hybrid male sterility, sequence divergence at genes involved in male reproduction is thought to be a major contributor to speciation [14]. Between the closely related species *D. simulans* and *D. mauritiana*, it is thought that ~60 loci contribute to hybrid male sterility [12]. To date, only a few of these loci have been mapped to the gene level [15–17]. For example, the first "speciation gene" identified between these two species, *OdsH*, encodes a homeodomain-containing transcription factor that is expressed in testis and shows extraordinary amino acid sequence divergence between *D. simulans* and *D. mauritiana* [15]. Within the homeodomain, 15 amino acids differ between these two

*Drosophila* species, while only 17 amino acids differ between mouse and common ancestor of the *D. melanogaster* clade [15]. These findings suggested that the early stages of speciation are driven by the rapid adaptive evolution of genes involved in male reproduction [18]. Consistent with this, it has been found that genes known to be involved in male reproduction, but not directly implicated in reproductive isolation between species, evolve at a faster rate than other classes of genes in the genome [19–21].

With the advent of transcriptomic technologies, such as microarrays, it became possible to examine gene expression differences between males and females on a genomic scale. In *Drosophila*, a large fraction of genes differ in expression between the sexes [22]. Such genes are referred to as "sex-biased." A meta-analysis over multiple experiments indicates that there are ~4,000 genes that show a large (greater than twofold) difference in expression between males and females of *D. melanogaster*, with ~2,000 showing male-biased expression and ~2,000 showing female-biased expression [23]. When statistical approaches are used to detect significant expression differences between the sexes, the number of sex-biased genes is even greater. For example, a meta-analysis with a false discovery rate of 5% classified 2,814 genes as male-biased and 4,056 genes as female-biased [23].

On average, male-biased genes display a faster rate of molecular evolution between species (as measured by the ratio of the nonsynonymous-to-synonymous substitution rates, $d_N/d_S$) than female-biased genes or genes with nearly equal expression in the two sexes ("unbiased genes") [23, 24]. By comparing levels of polymorphism within species to divergence between species, it could be shown that male-biased genes undergo more adaptive evolution than female-biased or unbiased genes [25]. This pattern was especially pronounced on the X chromosome, where X-linked, male-biased genes show exceptionally high $d_N/d_S$ and the strongest signal of adaptive protein evolution [26]. Although species outside the *melanogaster* species subgroup have not been investigated as extensively, preliminary studies in *D. ananassae* and *D. pseudoobscura* suggest that male-biased expression does not have as much of an influence on evolutionary rate in these species as it does in *D. melanogaster* [27–29].

In this paper, we examine the molecular divergence of sex-biased genes within and between species using data from *D. melanogaster* and *D. ananassae*. We also investigate intra- and interspecific divergence at the level of gene expression. Our results indicate that much of the protein divergence observed between species is adaptive. Male-biased genes of *D. melanogaster*, especially those that reside on the X chromosome, show an exceptionally high rate of adaptation. However, these genes do not show unusually high sequence variation within or between populations. At the level of gene expression, we find that both male- and female-biased genes make a large contribution to expression differences between species but are underrepresented among genes that differ in expression between populations. These findings suggest that different selective forces contribute to interpopulation and interspecies divergence.

Table 1: Numbers of genes analyzed.

| Species | Bias | Autosomal | X-linked |
|---|---|---|---|
| *D. melanogaster* | Male | 35 | 18 |
| | Unbiased | 32 | 16 |
| | Female | 29 | 13 |
| *D. ananassae* | Male | 10 | 7 |
| | Unbiased | 9 | 5 |
| | Female | 10 | 2 |

## 2. Materials and Methods

*2.1. D. melanogaster Genes and Populations.* In total, we analyzed DNA sequence polymorphism in 143 *D. melanogaster* genes (see Table 1 in Supplementary Material available online at doi:10.1155/2012/963976), which were classified as male-, female-, or unbiased in their expression using the Sebida database [23]. The numbers of sex-biased genes, as well as the numbers of X-linked and autosomal genes, are given in Table 1. All of the genes were sequenced in a sample of isofemale lines from two populations, one from Europe (Leiden, the Netherlands) and one from Africa (Lake Kariba, Zimbabwe) [34]. The number of alleles sequenced per population ranged from 7 to 12, with a mean of 11. Sequences of 136 of these genes were reported previously [24, 25, 35] and are available from the GenBank/EMBL databases under accession numbers AM293861–AM294919, AM998825–AM999334, and FM244915–FM246454. In addition, seven genes were newly sequenced for the current study and are available under accession numbers JN252131–JN252193 and JN374903–JN374992. For divergence calculations, a single allele from *D. simulans* was used [30].

*2.2. D. ananassae Genes and Populations.* For *D. ananassae*, we surveyed polymorphism in 43 genes (Supplementary Table 1), which were classified as male-, female-, or unbiased in their expression using data from a custom amplicon microarray [29] and a whole genome microarray analysis [36]. The 43 genes were a subset of those analyzed in *D. melanogaster*. The numbers of sex-biased genes, as well as the numbers of X-linked and autosomal genes, are given in Table 1. All of the genes were sequenced in a sample of isofemale lines from Bangkok, Thailand [29], and the sequences are available from the GenBank/EMBL databases under accession numbers FN546265–FN546780. The number of alleles sequenced ranged from 8 to 12, with a mean of 11. To calculate divergence, a single allele from either *D. atripex* or *D. phaeopleura* was used. The phylogenetic relationship of the species is shown in Figure 1.

*2.3. DNA Sequencing.* Genomic DNA was purified from single male flies, and target genes were PCR-amplified using protocols, primers, and cycling conditions described previously [25, 26]. Following PCR, the amplified products were purified with ExoSAP-IT (USB, Cleveland, OH, USA), and both strands were sequenced using BigDye version 1.1

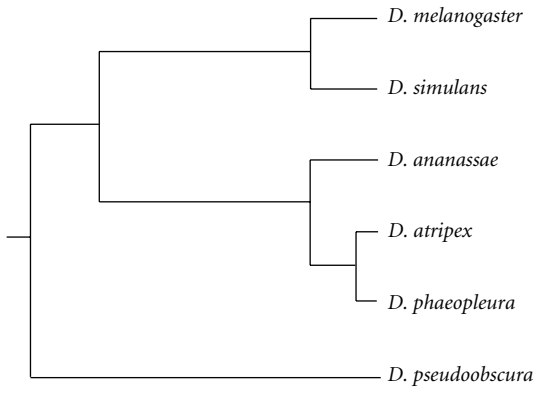


Figure 1: Phylogenetic relationship of the species used in this study [29, 30].

chemistry and a 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were edited using DNASTAR (Madison, WI, USA) and multiple alignments were generated using MUSCLE [37].

*2.4. Statistical Methods.* Standard polymorphism and divergence statistics were calculated using DnaSP version 5 [38]. To assess the significance of differences between sex-bias classes, the Kruskal-Wallis tests and the Mann-Whitney *U* tests were performed using R version 2.12.2 [39].

The distribution of fitness effects of new nonsynonymous mutations and the proportion of adaptive amino acid replacements between species, $\alpha$, were estimated using the DoFE software [40]. For this, the shape parameter was set to 0.5 and the number of repetitions for the MCMC chain was set to 5,000,000. Prior to running, a look-up table was generated, setting the upper limit of $\beta$ to 1 and the number of steps to 200. Otherwise, the default parameters were used. Synonymous sites were used as the neutral reference. This method requires the same sample size (number of sequences) for all genes. For *D. melanogaster*, we used a common sample size of 10 sequences from the African population. When more than 10 sequences were available for a gene, we randomly excluded surplus sequences. Genes with fewer than 10 sequences were excluded from the analysis. For *D. ananassae*, the above procedure was followed, but a common sample size of eight sequences was used.

## 3. Results

*3.1. Data.* In total, we analyzed DNA sequence polymorphism and divergence in 143 *D. melanogaster* and 43 *D. ananassae* protein-coding genes. Within each species, the genes could be assigned to one of three expression classes (male-, female-, or unbiased) on the basis of microarray data (Table 1) [23, 29, 36]. The proportion of genes in each expression class was similar, although there was a slight over-representation of male-biased genes. The genes could further be separated into those residing on the X chromosome and those residing on the autosomes (Table 1). For *D. melanogaster*, approximately one-third of the genes within each sex-bias class were X-linked. This allowed us to perform additional analyses in which X-linked and autosomal genes were considered separately within each expression class. Because the *D. ananassae* sample size was much smaller, we did not analyze X-linked and autosomal genes separately.

*3.2. Selective Constraint on Sex-Biased Genes.* To infer selective constraints, we used the method of Eyre-Walker and Keightley [40], which estimates the distribution of fitness effects of nonsynonymous mutations. In both *D. melanogaster* and *D. ananassae*, we found evidence for strong constraint on male-, female-, and unbiased genes, with the vast majority (>85%) of new mutations having a strongly deleterious effect, in which the product of the effective population size and the selection coefficient ($N_e s$) is greater than 10 (Figure 2). Less than 10% of mutations fell within the neutral range ($0 < N_e s < 1$). The level of constraint was similar across all classes of genes and in both species.

When the X-linked and autosomal genes of *D. melanogaster* genes were analyzed separately, there was again evidence for the predominance of strong purifying selection in all classes of genes (Figure 3). For male-biased and unbiased genes, there was a trend towards less constraint on the X chromosome. This pattern was not seen for female-biased genes.

*3.3. Adaptive Evolution of Sex-Biased Genes.* In both the *melanogaster* and *ananassae* lineages, we found that positive selection has made an important contribution to protein sequence divergence between species. For all classes of genes, the estimated proportion of adaptive nonsynonymous substitutions, $\alpha$, ranged from 0.29 to 0.83 (Figure 2). The 95% confidence interval of $\alpha$ excluded zero in all cases, except for the unbiased genes of *D. ananassae* where it was $-0.04$ to 0.56. In *D. melanogaster*, male-biased genes had the highest mean $\alpha$ and its 95% confidence interval did not overlap with that of female-biased or unbiased genes, indicating a significantly greater proportion of adaptive substitutions in male-biased genes. This pattern was not seen for *D. ananassae*, where $\alpha$ was highest for female-biased genes (Figure 2), but the 95% confidence intervals of $\alpha$ overlapped among all classes of genes.

Because the *D. ananassae* genes represented only a subset of those analyzed in *D. melanogaster*, it is possible that the observed differences in sex-biased gene evolution between species are a result of differences in gene composition or of limiting the *D. ananassae* genes to those that are well conserved and have identifiable orthologs in *D. melanogaster*. To examine these possibilities, we repeated our *D. melanogaster* analyses using only genes common to both species' gene sets (Figure 4(a)) or only genes with identifiable orthologs between species (Figure 4(b)). In both cases, we still observed higher values of $\alpha$ for male-biased genes than for female-biased or unbiased genes. For the set of common genes, which had a small sample size (37 genes total), the 95% confidence intervals of $\alpha$ overlapped among all classes of genes. However, for the set of genes with orthologs (108 genes total), the 95% confidence interval of $\alpha$ of male-biased genes did not overlap with that of female-biased or unbiased genes.

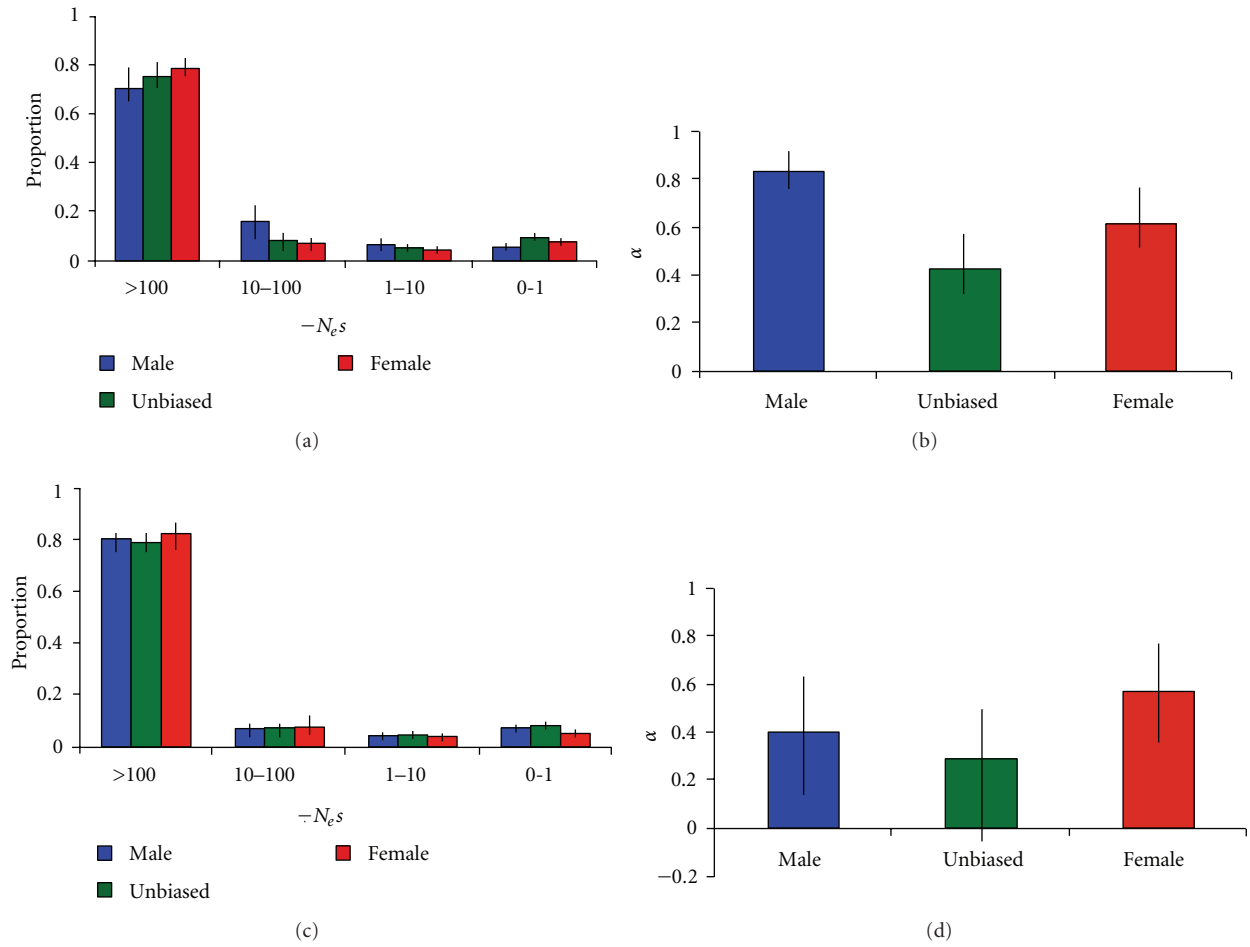


Figure 2: Distribution of fitness effects for nonsynonymous mutations within species and the proportion of adaptive nonsynonymous substitutions between species. Data for *D. melanogaster* are shown in (a) and (b), while those for *D. ananassae* are shown in (c) and (d).

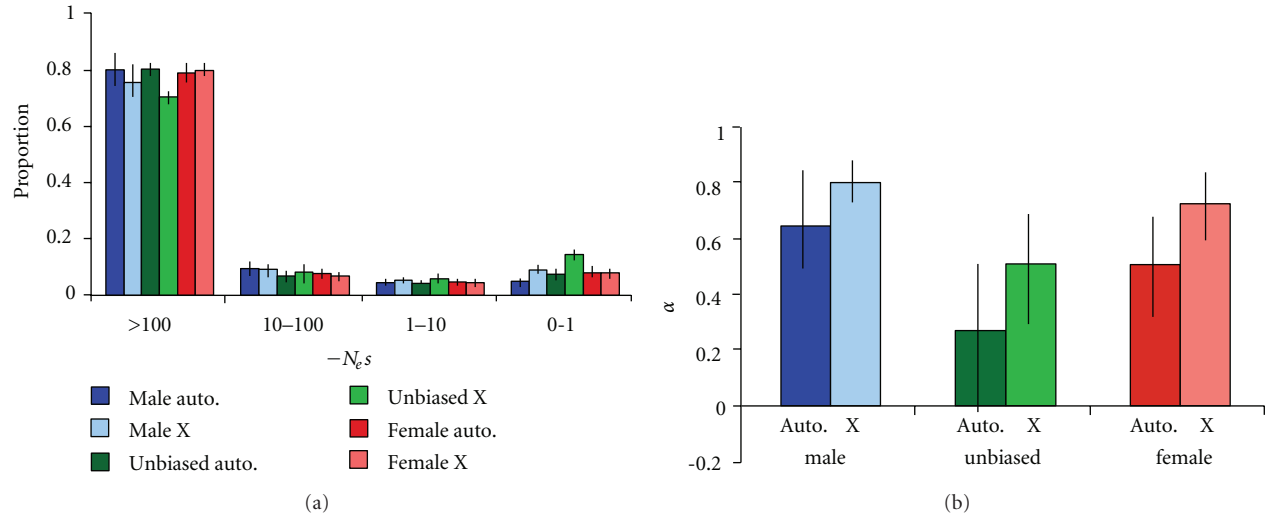


Figure 3: The distribution of fitness effects (a) and estimated proportion of adaptive substitutions (b) for autosomal and X-linked genes of *D. melanogaster*.

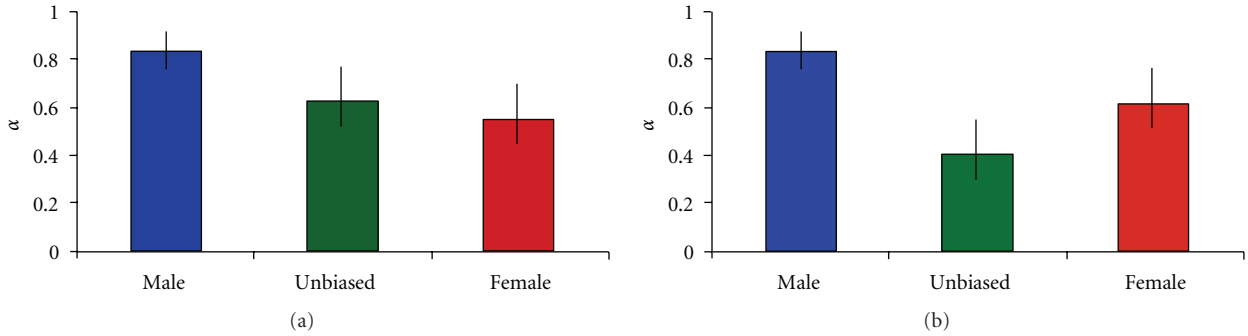


Figure 4: The estimated proportion of adaptive substitutions for *D. melanogaster* genes also present in the *D. ananassae* gene set (a) and for *D. melanogaster* genes that have an ortholog in *D. ananassae* (b).

Table 2: Rates of adaptive substitution in *D. melanogaster* and *D. ananassae*.

| Species | Bias | Chrom. | $d_N$ [a] | $\alpha d_N$ [b] |
|---|---|---|---|---|
| *D. melanogaster* | Male | Auto. | 14.0 | 9.1 |
| | | X | 60.8 | 49.8 |
| | Unbiased | Auto. | 8.3 | 2.3 |
| | | X | 24.6 | 12.5 |
| | Female | Auto. | 16.1 | 8.3 |
| | | X | 24.1 | 17.7 |
| *D. ananassae* | Male | Auto. + X | 17.7 | 7.1 |
| | Unbiased | Auto. + X | 15.9 | 4.6 |
| | Female | Auto. + X | 17.4 | 10.0 |

[a] Nonsynonymous substitutions per 1,000 nonsynonymous sites.
[b] Adaptive nonsynonymous substitutions per 1,000 nonsynonymous sites.

This indicates that the increased level of adaptive evolution of male-biased genes in *D. melanogaster* is not attributable to the rapid evolution of young, newly evolved genes that lack orthologs in *D. ananassae*.

When *D. melanogaster* autosomal and X-linked genes were considered separately, there was a consistent pattern of higher $\alpha$ for X-linked genes of all classes, with the highest value observed for male-biased, X-linked genes (Figure 3). This pattern was even more pronounced when the nonsynonymous substitution rate was taken into account, as X-linked genes showed greater nonsynonymous divergence (Table 2).

*3.4. Sequence Variation of Sex-Biased Genes within Populations.* Mean levels of nucleotide diversity ($\pi$) did not differ significantly among male-, female-, or unbiased genes in the Zimbabwe population of *D. melanogaster* or the Bangkok population of *D. ananassae* (Figure 5). This result held regardless of whether synonymous diversity ($\pi_S$), nonsynonymous diversity ($\pi_N$), or their ratio ($\pi_N/\pi_S$) was considered.

When *D. melanogaster* X-linked genes were considered separately, there was a significant difference in $\pi_N$ among male-, female-, and unbiased genes (the Kruskal-Wallis test, $P = 0.03$). This was mainly a result of X-linked, unbiased genes having high nonsynonymous diversity (Figure 6). There were no significant differences in $\pi_S$, $\pi_N$, or $\pi_N/\pi_S$ among autosomal male-, female-, or unbiased genes (the Kruskal-Wallis test, $P > 0.20$ in all cases). Within expression classes, there was consistently greater polymorphism at X-linked loci than at autosomal loci (Figure 6). This difference was significant only for unbiased genes, where $\pi_N$, and $\pi_N/\pi_S$ were both greater on the X chromosome than the autosomes (the Mann-Whitney test, $P = 0.002$ and $P = 0.006$, resp.).

*3.5. Sequence Divergence of Sex-Biased Genes between Populations.* For *D. melanogaster*, we had sequence data for all 143 genes from both an African (Zimbabwe) and a European (the Netherlands) population, which allowed us to determine the contribution of sex-biased genes to interpopulation genetic differentiation. Two measures, $F_{ST}$ and $D_{XY}$ (the mean number of pairwise sequence differences between alleles of the two populations), indicated that there are similar levels of differentiation for male-, female-, and unbiased genes on both the X chromosome and the autosomes (Table 3). However, for all classes of genes, differentiation was greater at X-linked loci. For male- and female-biased genes, $F_{ST}$ was significantly greater on the X chromosome when all sites or only synonymous sites were considered (Table 3). For unbiased genes, $D_{XY}$ was significantly greater on the X chromosome for nonsynonymous sites (Table 3).

*3.6. Intra- and Interspecific Divergence in Sex-Biased Gene Expression.* To determine the contribution of sex-biased genes to variation within and between species at the level of gene expression, we analyzed data from published microarray studies that investigated expression polymorphism within *D. melanogaster* [31, 32] and expression divergence between *D. melanogaster* and *D. simulans* [33]. Three types of expression variation were examined (intrapopulation, interpopulation, and interspecies) using data from males and females separately (Figure 7). When expression was measured in males, male-biased genes showed the highest levels of intrapopulation and interspecies divergence. However, male-biased genes did not show increased expression divergence between populations. When measured in females, female-biased genes showed the least intrapopulation and interpopulation expression polymorphism, but the greatest interspecies expression divergence.

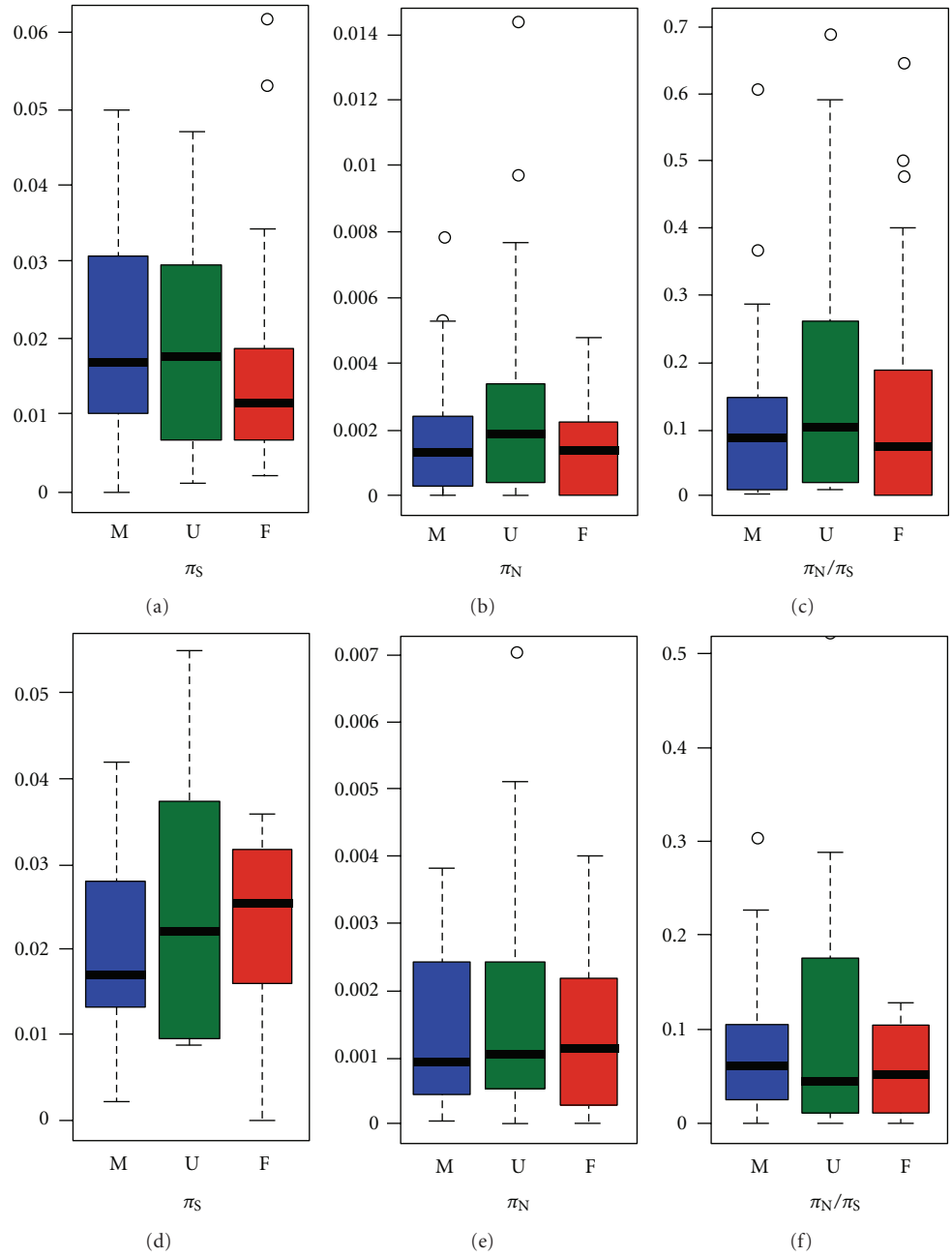


FIGURE 5: Intraspecies polymorphism in male-biased (M), unbiased (U), and female-biased (F) genes of *D. melanogaster* (a–c) and *D. ananassae* (d–f). Shown are distributions of synonymous nucleotide diversity ($\pi_S$), nonsynonymous nucleotide diversity ($\pi_N$), and their ratio ($\pi_N/\pi_S$). The *D. melanogaster* data are from the African (Zimbabwe) population. There were no significant differences among male-, female-, and unbiased genes in either species by any measure (the Kruskal-Wallis test, $P > 0.10$ in all cases).

## 4. Discussion

*4.1. Selection on Sex-Biased Genes.* Our analyses of polymorphism and divergence in *D. melanogaster* and *D. ananassae* uncovered several common patterns. First, there is strong purifying selection acting at the protein level in both species. We estimate that over 85% of all newly arising nonsynonymous mutations are deleterious. Second, a large proportion of amino acid substitutions that have become fixed between species can be attributed to positive selection. Our estimates of $\alpha$ range from 27 to 83% in *D. melanogaster* and 29–57% in *D. ananassae*. In *D. melanogaster*, male-biased genes showed the highest values of $\alpha$ (Figure 2), which is consistent with previous studies [25, 26]. In *D. ananassae*, there was no evidence for increased adaptive evolution of male-biased genes, which suggests that there are differences in sex-biased gene evolution among lineages[27, 29].

Our estimates of $\alpha$ are in line with previously published estimates and suggest that adaptive protein evolution is widespread across the *Drosophila* genus [41]. A recent study

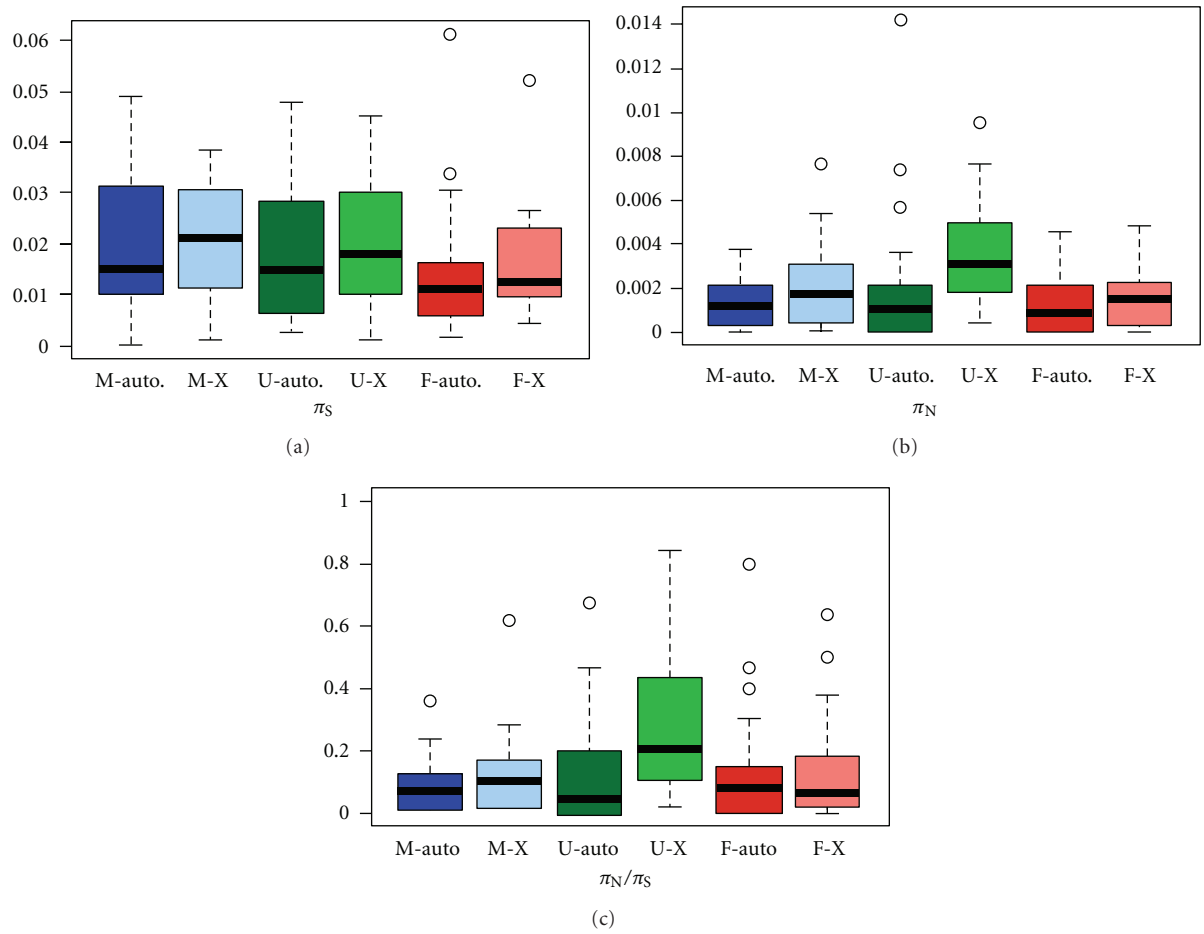


FIGURE 6: Intraspecies polymorphism in autosomal and X-linked genes of *D. melanogaster*. Shown are distributions of synonymous nucleotide diversity ($\pi_S$), nonsynonymous nucleotide diversity ($\pi_N$), and their ratio ($\pi_N/\pi_S$). The data are from the African (Zimbabwe) population. The only significant difference among expression classes was for $\pi_N$ (the Kruskal-Wallis test, $P = 0.03$), where X-linked unbiased genes had significantly higher $\pi_N$ than X-linked female-biased genes (the Mann-Whitney test, $P = 0.01$). Within expression classes, X-linked unbiased genes had significantly greater $\pi_N$ and $\pi_N/\pi_S$ than autosomal unbiased genes (the Mann-Whitney test, $P = 0.002$ and $P = 0.006$, resp.).

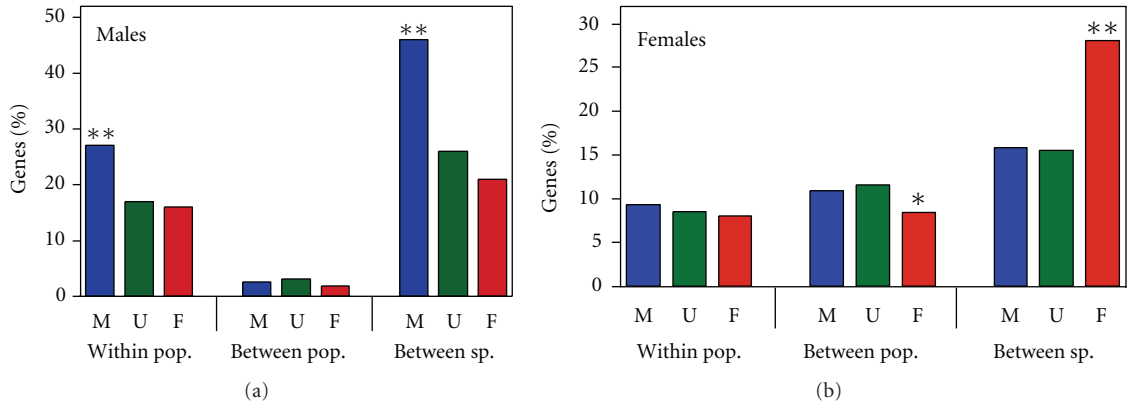


FIGURE 7: Gene expression variation within a population, between populations, and between species. Shown are the percentages of male-biased (M), unbiased (U), and female-biased (F) genes that show significant expression variation within a Zimbabwe population of *D. melanogaster*, between a Zimbabwe and a European population of *D. melanogaster* and between *D. melanogaster* and *D. simulans*. Expression variation was measured separately in males (a) and females (b). Data are from Hutter et al. [31], Müller et al. [32], and Ranz et al. [33]. Asterisks indicate significant differences from unbiased genes within the same comparison, as determined by Fisher's exact test. $^*P < 0.001$, $^{**}P < 0.0001$.

Table 3: Mean $F_{ST}$ and $D_{XY}$ between the African and European *D. melanogaster* populations.

| Sites[a] | Bias[b] | Autosomal | | X-linked | |
|---|---|---|---|---|---|
| | | $F_{ST}$ (SD) | $D_{XY}$[c] (SD) | $F_{ST}$ (SD)[d] | $D_{XY}$[c] (SD)[d] |
| All | M | 0.157 (0.142) | 0.69 (0.35) | 0.266 (0.141)** | 0.72 (0.37) |
| | U | 0.160 (0.125) | 0.74 (0.60) | 0.231 (0.231) | 0.87 (0.48) |
| | F | 0.195 (0.161) | 0.56 (0.41) | 0.343 (0.186)* | 0.59 (0.30) |
| Syn | M | 0.164 (0.164) | 2.25 (1.87) | 0.261 (0.151)* | 2.68 (2.60) |
| | U | 0.159 (0.139) | 1.94 (1.63) | 0.223 (0.020) | 2.57 (1.56) |
| | F | 0.185 (0.163) | 1.54 (1.23) | 0.319 (0.209)* | 1.82 (1.27) |
| Non | M | 0.090 (0.086) | 0.14 (0.12) | 0.185 (0.199) | 0.27 (0.28) |
| | U | 0.107 (0.123) | 0.19 (0.27) | 0.128 (0.143) | 0.51 (0.80)** |
| | F | 0.149 (0.171) | 0.12 (0.15) | 0.254 (0.305) | 0.17 (0.17) |

[a] "All," all sites (including introns); "Syn," synonymous sites; "Non," nonsynonymous sites.
[b] "M," male-biased; "U," unbiased; "F," female-biased.
[c] Mean pairwise differences between all African and all European sequences (in %).
[d] Asterisks indicate significant differences from autosomal genes by the Mann-Whitney test. $^*P < 0.05$, $^{**}P < 0.01$.

of *D. melanogaster* and *D. simulans* reported higher estimates of $\alpha$ for a randomly chosen (with respect to expression and function) set of genes [42]. However, this study was limited to X-linked genes, which tend to have higher values of $\alpha$ (Figure 3). This suggests that the use of only X chromosomal data may lead to an overestimate of the genome-wide proportion of adaptive substitutions.

*4.2. Faster-X Evolution.* Several factors could contribute to the increased rate of adaptive evolution of X-linked genes. For example, the X chromosome could have a larger effective population size ($N_e$) than the autosomes. Assuming an equal sex ratio, the number of X chromosomes in a population is expected to be 75% that of the number of autosomes. However, sexual selection acting on males can lead to a reduction in the $N_e$ of the autosomes relative to the X chromosome, and this could accelerate X chromosome evolution [43, 44]. In our populations of *D. melanogaster* and *D. ananassae*, which are thought to come from the ancestral species ranges [45, 46], the X chromosome and the autosomes have nearly identical $N_e$ [29, 35, 47], making this explanation unlikely. Furthermore, there is no evidence for increased purifying selection on the X chromosome (Figure 3), which would be expected if it had a larger $N_e$. This observation also argues against the possibility that an increased rate of recombination on the X chromosome leads to an increase in the efficacy of selection on X-linked loci by reducing interference among mutations [48, 49].

The accelerated rate of adaptive evolution on the X chromosome could also be explained by an increased rate of fixation of new, beneficial, recessive mutations due to their exposure to selection in hemizygous males [50, 51]. This would explain why the signal of adaptive evolution is strongest for male-biased genes, as they are expected to encounter selection mainly in the male (hemizygous) genetic background [26]. Although female-biased genes would be expected to receive the least benefit from selection in the male genetic background, a recent study found that mutations in female-biased genes often have phenotypic effects in males [52]. Thus, the increased rate of adaptive evolution seen for X-linked, female-biased genes could result from their exposure to selection in males.

*4.3. Gene Expression Divergence.* Our analyses of gene expression polymorphism and divergence revealed that the genes with the greatest expression divergence between species are those that are expressed predominantly in the sex that is used for comparison. When males are compared, male-biased genes show the greatest interspecific difference in expression (Figure 7). When females are compared, female-biased genes show the greatest interspecific difference in expression. These patterns are not seen for interpopulation expression divergence, where male- and female-biased genes consistently show less interpopulation expression divergence than unbiased genes, regardless of the sex that is compared (Figure 7). Thus, similar to protein divergence, gene expression divergence between species does not appear to be a simple extension of divergence between populations.

There are some caveats to our expression analysis. First, the experiments were performed by different groups, at different times, and with different microarray platforms. Thus, many factors may contribute to the differences seen among experiments. However, this problem will not apply to comparisons of male-, female-, and unbiased genes within each experiment, as all of the genes were measured together on the same microarrays. Thus, we expect comparisons of sex-biased genes within experiments to be robust. A second caveat is that the interspecies comparisons used only a single isofemale line of each species. This means that intraspecific polymorphism and interspecific divergence will be confounded. However, given the low level of expression polymorphism seen within species, it is unlikely that intraspecific gene expression polymorphism has much influence on measures of interspecific divergence. This is supported by the observation that, in females, there is no correspondence between the relative levels of expression polymorphism and divergence (Figure 7). However, for the experiments performed on males, we cannot exclude the possibility that the observed interspecific divergence of male-biased genes is inflated by intra-specific polymorphism.

*4.4. Implications for Speciation.* Although it is not possible to establish a direct link between sex-biased gene evolution and speciation in most cases, several of our observations are consistent with the rapid evolution of sex-biased genes (especially male-biased genes) contributing to reproductive isolation, at least for *D. melanogaster* and its close relatives. The male-biased genes examined here are expressed in reproductive tissues [26], and their rapid adaptive evolution could contribute to the male-hybrid sterility that is often seen as a first step in reproductive isolation. Furthermore, the rapid adaptive evolution of X-linked genes, especially those with male-biased expression, is consistent with the disproportionately large effect that the X chromosome has on hybrid breakdown [12, 13]. At the level of gene expression, male-biased genes make the largest contribution to the expression differences between species in males. Since the vast majority of male-biased genes are expressed in reproductive tissues [53], it is likely that expression differences also contribute to male hybrid sterility and other forms of hybrid breakdown.

## Acknowledgments

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*Research Article*

# Differences in Chemical Sexual Signals May Promote Reproductive Isolation and Cryptic Speciation between Iberian Wall Lizard Populations

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Interpopulational variation in sexual signals may lead to premating reproductive isolation and speciation. Genetic and morphological studies suggest that the Iberian wall lizard, *Podarcis hispanica*, forms part of a "species complex" with several cryptic species. We explored the role of chemical sexual signals in interpopulational recognition between five distinct populations of Iberian wall lizards in Central Spain. Results showed that these populations differed in morphology and in composition and proportion of chemical compounds in femoral gland secretions of males. Tongue-flick experiments indicated that male and female lizards discriminated and were more interested in scents of lizards from their own area (i.e., Northern versus Southern populations), but did not discriminate between all populations. Moreover, only males from the populations that are geographically located more far away preferred scent of females from their own population. These data suggest that, at least between some populations, there may be reproductive isolation mediated by chemical signals and cryptic speciation.

## 1. Introduction

Interpopulational variation in sexual chemical signals may provide the basis for premating reproductive isolation and speciation in many animals [1, 2]. Phenotypic plasticity in sexual signals could play a key role in initial signal divergence [3], for example, as a way to maximize the efficiency of signals for communication in different environments [4, 5]. These differences can be later amplified by sexual selection leading to differences in mating preferences [6–8], which could preclude mating between populations (e.g., [9–13]), and lead to speciation processes.

In many lizards, intraspecific communication and sexual selection are based on chemical signals secreted by specific glands [14–17]. For example, chemical compounds secreted by femoral gland of males can convey information about social status [18–22] and genetic quality of a male [23–26]. Also, differences in chemical signals may preclude interspecific mating between related sympatric species (e.g., [27, 28]). We hypothesized that interpopulational variations in femoral gland secretions within the same species might lead to reproductive isolation and thus promote speciation processes.

The Iberian wall lizard, *Podarcis hispanica*, is a small diurnal lizard, living in rocky habitats of the Iberian Peninsula. Molecular and morphological studies suggest that this lizard is paraphyletic and forms part of a "species complex," which suggests the existence of cryptic speciation within taxa previously considered to be conspecific [29–34]. Chemosensory recognition is well developed in *P. hispanica* [11, 35]. This lizard can discriminate between sexes by chemical cues alone [36–39]. Chemical cues of males, mainly from the femoral gland secretions, are important in male-male interactions [19, 20, 40] and in female mate choice decisions [35, 41, 42]. Also, chemical cues of females, in conjunction with coloration, elicit courtship by males [37]. At least two populations differ in chemical characteristics of femoral secretions of males [11]. This raises the possibility that

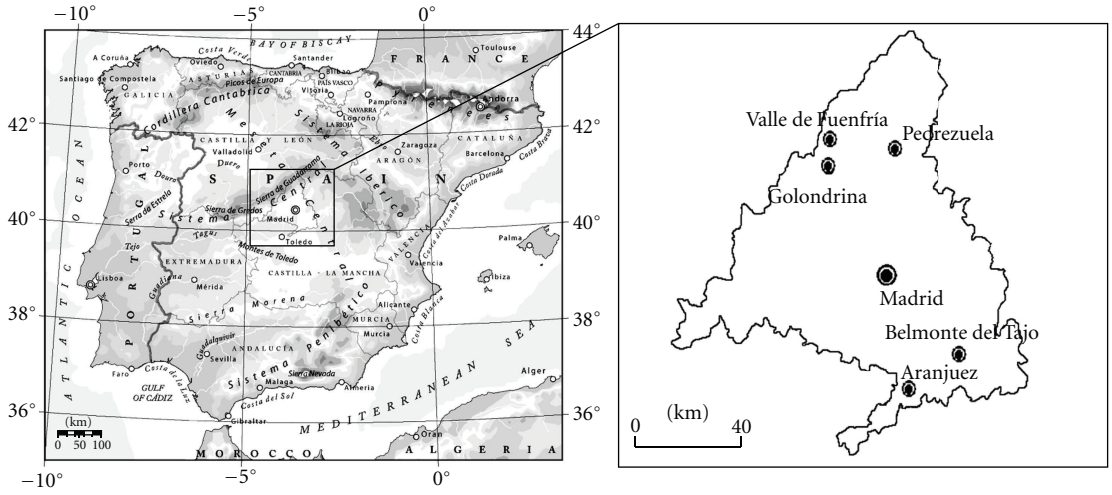


Figure 1: Geographic localization of the five populations of *Podarcis hispanica* studied in the Madrid region in the center of Spain.

*P. hispanica* lizards use chemical sexual signals to discriminate between populations, which might lead to reproductive isolation (if variation of signals is discrete or there is a barrier to gene flow) and explain the genetic and morphological differences observed between populations.

In this study, we explored the role of chemical sexual signals in interpopulational recognition between five distinct populations of Iberian wall lizards in Central Spain. In this area, several populations inhabiting different environments live close together without geographical barriers that isolate the populations, and individuals may find each other easily [11, 12, 43]. However, some populations maintain clear distinct morphotypes and differ genetically [32–34], which suggests that they might be, at least partly, reproductively isolated. We hypothesized that interpopulational variations in chemical signals could allow chemosensory recognition between populations and lead to premating boundaries. To test this, we first compared the morphological characteristics of these populations, and then we analyzed whether there was variation in the composition and proportions of chemical compounds in femoral gland secretions of males by using gas chromatography-mass spectrometry (GC-MS). We further conducted tongue-flick experiments to analyze whether males and females discriminated by chemosensory cues alone between scent of lizards from different populations. We hypothesized that male and female lizards could be able to recognize by chemical cues alone, and maybe prefer, the scents of individuals of their own population, which may contribute to a reduced gene flow. We expected that interpopulational differences in chemical signals of males and in population recognition abilities could suggest the probable existence of reproductive isolation and cryptic speciation between these Iberian wall lizard populations.

## 2. Methods

*2.1. Study Populations.* During February-March 2008, we captured by noosing male and female *P. hispanica* lizards at five localities within the Madrid Region (Central Spain) (Figure 1). Three of these were localized in the Northern mountain area ("Fuenfría," "Golondrina," and "Pedrezuela"), and the other two were situated in the Southern plain area ("Belmonte" and "Aranjuez"). We selected these populations because lizards clearly differ in morphology and coloration [43, 44]. In the North, we captured lizards from a population occupying different granite rock cliffs at the edge of a pine forest in the upper part of the "Fuenfría" Valley (40°47′N, 04°03′W; 1750 m altitude; 21 males and 26 females), on granite rocky outcrops inside a large oak forest "Golondrina" near Cercedilla village (40°44′N, 04°02′W; 1250 m altitude; 29 males and 27 females), and from old stone walls near crop fields close to "Pedrezuela" village (40°44′N, 03°36′W; 800 m altitude; 19 males and 16 females). In the South, we captured lizards on human buildings and walls inside a public garden of the "Belmonte del Tajo" village (40°08′N, 03°20′W; 735 m altitude; 22 males and 17 females) and on chalk and gypsum rocks in deforested bushy hills near "Aranjuez" village (40°02′N, 03°37′W; 494 m altitude; 21 males and 32 females).

All lizards were individually housed at "El Ventorrillo" Field Station (Cercedilla, Madrid) about 5 Km from the capture sites of the Northern populations, in indoor 60 × 40 cm PVC terraria containing sand substratum and rocks for cover. Cages were heated with 40 W spotlights during 6 h/day, and overhead lighted (36 W full-spectrum daylight tubes) on a 10 h : 14 h light/dark cycle, and were screened from each other using cardboard. Every day, lizards were fed mealworm larvae (*Tenebrio molitor*) dusted with multivitamin powder for reptiles, and water was provided *ad libitum*. Lizards were returned to their exact capture sites with good health condition at the end of experiments.

*2.2. Morphological Characteristics.* We made the following morphological measurements of each individual lizard: body mass (or weight) (measured with a digital balance to the nearest 0.01 g) and body size (snout-to-vent length, SVL;

measured with a ruler to the nearest 1 mm). We also made morphological measurements of the head using a digital caliper (to the nearest 0.05 mm). Head length was the distance between the tip of the snout and the posterior side of the parietal scales. Head width was the greatest distance between the external sides of the parietal scales. Head depth was the greatest distance from the highest portion of the head to the bottom of the lower jaw.

We also counted under a magnifying glass the number of femoral pores on the right and left hindlimbs of lizards and calculated an average number for both sides. Finally, we noted the number of small but distinctive and conspicuous blue ocelli that runs along each of the body sides on the outer margin of the belly of males and calculated an average number for both sides. These ocelli seem to have a role in sex recognition and intrasexual social relationships between males [39, 45].

All biometrical variables were log transformed prior to analysis to meet assumptions of normality and homoscedasticity. We used one-way analyses of variance (ANOVAs) to test for differences in morphological variables between populations. Pairwise comparisons were based on Tukey's honestly significant difference (HSD) tests [46].

*2.3. Chemical Analyses of Femoral Gland Secretions.* Immediately after capture in the field, we extracted femoral gland secretion of males by gently pressing with forceps around the femoral pores and collected secretion directly in glass vials with Teflon-lined stoppers. Vials were stored at −20°C until analyses. We also used the same procedure on each sampling occasion but without collecting secretion, to obtain blank control vials that were treated in the same manner to compare with the lizard samples. Before the analyses we added 250 $\mu$L of *n*-hexane (Sigma, capillary GC grade) to each vial. We analyzed lipophilic compounds in samples by using a Finnigan-ThermoQuest Trace 2000 gas chromatograph (GC) fitted with a poly (5% diphenyl/95% dimethylsiloxane) column (Thermo Fisher, Trace TR-5, 30 m length × 0.25 mm ID, 0.25 mm film thickness) and a Finnigan-ThermoQuest Trace mass spectrometer (MS) as detector. Sample injections (2 $\mu$L of each sample dissolved in *n*-hexane) were performed in splitless mode using helium as the carrier gas at 30 cm/sec, with injector temperature at 250°C. The oven temperature program was as follows: 50°C isothermal for 5 min, then increased to 270°C at a rate of 10°C/min, isothermal for 1 min, then increased to 315°C at rate of 15°C/min, and finally isothermal (315°C) for 10 min. Ionization by electron impact (70 eV) was carried out at 250°C. Mass spectral fragments below $m/z = 39$ were not recorded. Impurities identified in the solvent and/or the control vial samples are not reported.

Initial tentative identification of secretion components was done by comparison of mass spectra in the NIST/EPA/NIH 1998 computerized mass spectral library. Identifications were confirmed by comparison of spectra and retention times with those of authentic standards from Sigma-Aldrich Chemical Co. For unidentified or unconfirmed compounds we report here their characteristic ions, which we used together with retention times and characteristic $m/z$ ratios to confirm whether these compounds were present in a given individual.

For the statistical analyses of femoral secretions, the relative amount of each component was determined as the percent of the total ion current (TIC). The relative areas of the peaks were transformed following Aitchison's formula [47–49]. The homogeneity of variance of these variables was tested with Levene's test, and Bonferroni's correction was applied. The transformed areas were used as variables in a principal component analysis with varimax rotation. The eight principal components (PCs) extracted (all with eigenvalues >1, which explained 82.55% of variance) were used as covariates in a discriminant analysis to test whether chemical compounds in femoral secretions could be used to predict the population of origin of a male lizard. Then, we calculated the squared Mahalanobis distances of individuals with all other individuals and compared them between populations.

*2.4. Chemosensory Recognition between Populations.* Lizards have been shown to react to a variety of chemical stimuli with increased and differential rates of tongue extrusions [50]. Tongue-flick (TF) rate can, therefore, be used as a quantitative bioassay of detection of chemical cues (e.g., [11, 38]). Thus, to test for differential responses to scents, we made comparisons of TF rate by lizards (males and females) in response to chemical stimuli presented on cotton applicators impregnated with scents of male or female *P. hispanica* from each of the five different populations (Aranjuez, Golondrina, Fuenfría, Pedrezuela, and Belmonte) or with deionized water (odorless control). Water was used to gauge baseline TF's rates in the experimental situation [50]. We obtained lizard scents from the femoral pores of males or from the cloacal area of females because these are the body areas most frequently and intensely investigated by tongue flicking during social encounters [19, 37, 39]. Therefore, after first dipping the cotton tip (1 cm) of a wooden applicator attached to a long stick (50 cm) in deionized water, we rolled the tip over those body areas (of one population and sex per applicator). We used a new applicator in each trial.

First, males were exposed to scents from males and then to scents from females of each population tested. Finally we studied the responses of females to scent of males of each population. Every lizard was exposed to each stimulus and order of presentation was counterbalanced. One trial was conducted per day for each animal. Trials were conducted in outdoor conditions during April, which coincided with the mating season of lizards in their original natural populations (P. López and J. Martín, unpublished data), and between 11:00 and 13:00 (GMT) when lizards were fully active.

To begin a trial, the experimenter slowly approached the terrarium and slowly moved the cotton swab to a position 1 cm anterior to the lizards' snout. Lizards usually did not flee from the swab, but explore it repeatedly by tongue flicking or ignore it after the first TFs. In all cases, lizards directed TFs to the swab in all conditions. The numbers of TFs directed at the swab were recorded for 60 s

Table 1: Morphological characteristics (mean ± SE) of *P. hispanica* lizards (males and females) from five distinct populations of the Madrid region (Aranjuez, Belmonte, Golondrina, Fuenfría, and Pedrezuela). Results from one-way ANOVAs comparing morphological measures between populations are shown. The same letter after the means denotes a nonsignificant difference in post hoc tests.

| Morphological measures | Populations | | | | | ANOVAs | |
|---|---|---|---|---|---|---|---|
| | Aranjuez | Belmonte | Golondrina | Fuenfría | Pedrezuela | $F_{4,107}$ | $P$ |
| *Males* | | | | | | | |
| Weight (g) | $3.3 \pm 0.2^{ab}$ | $3.1 \pm 0.2^{a}$ | $4.7 \pm 0.2^{bc}$ | $5.5 \pm 0.2^{c}$ | $3.8 \pm 0.2^{b}$ | 35.89 | < 0.001 |
| SVL (mm) | $51 \pm 1^{ab}$ | $50 \pm 1^{a}$ | $59 \pm 1^{bc}$ | $62 \pm 1^{c}$ | $55 \pm 1^{b}$ | 38.80 | < 0.001 |
| Head length (mm) | $12.7 \pm 0.2^{a}$ | $12.4 \pm 0.2^{ac}$ | $14.2 \pm 0.2^{bc}$ | $14.9 \pm 0.1^{b}$ | $13.8 \pm 0.2^{c}$ | 16.57 | < 0.001 |
| Head width (mm) | $7.3 \pm 0.1^{a}$ | $7.5 \pm 0.1^{ac}$ | $8.2 \pm 0.1^{bc}$ | $8.5 \pm 0.1^{b}$ | $8.0 \pm 0.1^{c}$ | 11.96 | <0.001 |
| Head depth (mm) | $5.3 \pm 0.1^{a}$ | $5.6 \pm 0.1^{ac}$ | $6.1 \pm 0.1^{bc}$ | $6.1 \pm 0.1^{b}$ | $5.9 \pm 0.1^{c}$ | 31.20 | <0.001 |
| Femoral pores | $16.2 \pm 0.2^{a}$ | $17.5 \pm 0.3^{b}$ | $18.3 \pm 0.3^{b}$ | $17.7 \pm 0.3^{b}$ | $17.2 \pm 0.3^{ab}$ | 7.12 | <0.001 |
| Blue ocelli | $4.5 \pm 0.5^{a}$ | $4.9 \pm 0.5^{a}$ | $1.9 \pm 0.5^{b}$ | $1.7 \pm 0.4^{b}$ | $5.9 \pm 0.6^{a}$ | 13.41 | <0.001 |
| *Females* | | | | | | $F_{4,113}$ | $P$ |
| Weight (g) | $2.9 \pm 0.1^{ab}$ | $2.5 \pm 0.1^{a}$ | $2.9 \pm 0.1^{ab}$ | $3.3 \pm 0.2^{b}$ | $2.8 \pm 0.2^{ab}$ | 3.20 | 0.015 |
| SVL (mm) | $50 \pm 1^{ab}$ | $50 \pm 1^{ab}$ | $55 \pm 1^{bc}$ | $56 \pm 1^{c}$ | $52 \pm 1^{b}$ | 11.43 | <0.001 |
| Head length (mm) | $11.0 \pm 0.1^{a}$ | $11.1 \pm 0.2^{a}$ | $11.8 \pm 0.1^{b}$ | $12.0 \pm 0.1^{b}$ | $11.5 \pm 0.2^{a}$ | 3.49 | 0.01 |
| Head width (mm) | $6.6 \pm 0.1^{a}$ | $6.5 \pm 0.1^{a}$ | $6.7 \pm 0.1^{b}$ | $6.9 \pm 0.1^{b}$ | $6.6 \pm 0.1^{a}$ | 3.48 | 0.01 |
| Head depth (mm) | $4.8 \pm 0.1^{a}$ | $4.8 \pm 0.1^{a}$ | $5.0 \pm 0.1^{b}$ | $5.1 \pm 0.1^{b}$ | $4.7 \pm 0.1^{a}$ | 9.40 | <0.001 |
| Femoral pores | $13.7 \pm 0.2^{a}$ | $16.4 \pm 0.3^{b}$ | $15.6 \pm 0.2^{b}$ | $16.0 \pm 0.2^{b}$ | $15.3 \pm 0.2^{ab}$ | 21.62 | <0.001 |

beginning with the first TF. Analyses were made separately for responding males and females. To examine differences among treatments, previous analyses showed that responses to the different scents differed as a function of the population of the responding lizard. Thus, we used separated one-way repeated measures ANOVAs to test the effect of scent stimuli (within factor; Fuenfría versus Golondrina versus Aranjuez versus Pedrezuela versus Belmonte versus water) in number of TFs directed at the swab within each population of responding lizards. Data were log-transformed to ensure normality. Tests of homogeneity of variances (Levene's test) showed that in all cases variances were not significantly heterogeneous after transformation. Pairwise comparisons were planned using Tukey's honestly significant difference (HSD) tests [46].

## 3. Results

*3.1. Interpopulational Differences in Morphology.* There were significant differences between populations in all morphological measurements (Table 1). In general, lizards from Fuenfría and Golondrina populations were significantly heavier and longer and had greater heads than lizards from Aranjuez and Belmonte, which did not differ. Lizards from Pedrezuela were intermediate in size between the other populations (Table 1). However, when the effect of variation in body size between populations was removed, head size differences were significant only for head depth (ANOVA on residuals of head size with SVL, $P = 0.005$ for both sexes), but not for head length ($P > 0.20$ for both) or width ($P > 0.05$ for both). With respect to the number of femoral pores, both male and female lizards from Aranjuez had significantly less femoral pores than lizards from Belmonte, Fuenfría, and Golondrina, which did not differ. Lizards from Pedrezuela had an intermediate number of pores (Table 1). The number of femoral pores was not significantly related to body size ($P > 0.60$ in all cases). Finally, males from Aranjuez, Belmonte, and Pedrezuela had significantly more blue ocelli than males from Fuenfría and Golondrina (Table 1).

*3.2. Interpopulational Differences in Chemical Composition of Femoral Secretions.* We found 53 lipophilic compounds in femoral gland secretions of male *P. hispanica* (Table 2). The lipophilic fraction of femoral gland secretions of males, all five populations pooled, is a mixture of steroids (83.69% of TIC), and carboxylic acids ranged between *n*-$C_{14}$ and *n*-$C_{22}$ and their esters (10.30%), but we found also five alcohols between *n*-$C_{16}$ and *n*-$C_{24}$ (3.53%), a furanone (1.18%), four waxy esters (1.10%), squalene (0.60%), and two terpenoids (0.28%). On average, the five most abundant chemicals were cholesterol (63.24% of TIC), followed by cholesta-5,7-dien-3-ol (5.16%), hexadecanoic acid (3.73%), campesterol (3.66%), octadecenoic acid (2.46%), and octadecanoic acid (1.77%). There were 34 chemical compounds shared by lizards from all populations, but we found differences between populations in the presence/absence of 19 compounds in femoral secretions (Table 2). The discriminant analysis showed that the eight PCs scores describing proportions of compounds in femoral secretions could be used to predict the population of origin of a male lizard (Wilks' $\lambda = 0.0001$, $F_{32,355} = 607.45$, $P < 0.0001$) (Figure 2). All the pairwise comparisons of the Mahalanobis distances between populations, which ranged between 150.35 and 1015.13, were significant in all cases ($210.46 < F_{8,96} < 1290.20$, $P < 0.0001$ in all cases).

*3.3. Chemosensory Responses of Males to Scent of Males.* The number of TFs differed significantly between the scents

Table 2: Lipophilic compounds found in femoral gland secretions of male lizards, *P. hispanica*, from five distinct populations of the Madrid region (Aranjuez, Belmonte, Golondrina, Fuenfría, and Pedrezuela). The relative amount of each component was determined as the percent of the total ion current (TIC) and reported as the average (±SE). Characteristics (*m/z*) are reported for some unidentified (Un.) compounds. (RT: retention time).

| Compounds | RT (min) | Fuenfría | Pedrezuela | Golondrina | Belmonte | Aranjuez |
|---|---|---|---|---|---|---|
| *Steroids* | | | | | | |
| Un. steroid (145,213,248,353,368,387) | 29.92 | 0.01 ± 0.01 | — | 0.17 ± 0.05 | 1.49 ± 0.56 | — |
| Cholesta-2-4-diene | 30.58 | 0.68 ± 0.11 | 2.66 ± 0.44 | 0.44 ± 0.08 | 2.59 ± 0.46 | 0.96 ± 0.35 |
| Cholesta-3.5-diene | 30.81 | 0.42 ± 0.10 | 0.23 ± 0.04 | 0.30 ± 0.07 | 0.13 ± 0.03 | 0.25 ± 0.07 |
| Un. steroid (155,197,251,350,365) | 30.96 | 1.32 ± 0.16 | 1.00 ± 0.14 | 0.55 ± 0 | 0.45 ± 0.06 | 0.45 ± 0.17 |
| Cholesta-5,7,9(11)-trien-3-ol | 31.06 | 1.62 ± 0.18 | 1.07 ± 0.24 | 0.94 ± 0.11 | 0.65 ± 0.11 | 0.29 ± 0.07 |
| Un. steroid (207,251,350,365) | 31.13 | 0.40 ± 0.08 | 0.16 ± 0.02 | 0.18 ± 0.02 | 0.18 ± 0.07 | 0.08 ± 0.04 |
| Un. steroid (143,195,207,351,366) | 31.20 | 0.19 ± 0.02 | 0.08 ± 0.01 | 0.15 ± 0.04 | 0.18 ± 0.05 | 0.22 ± 0.06 |
| Un. steroid (141,156,209,350,365) | 31.37 | 0.37 ± 0.05 | 0.03 ± 0.01 | 0.30 ± 0.06 | 2.47 ± 0.42 | — |
| Un. steroid (155,197,251,365,379) | 31.64 | 0.06 ± 0.01 | 0.21 ± 0.02 | 0.08 ± 0.02 | 0.43 ± 0.07 | 0.45 ± 0.18 |
| Un. steroid (195,209,251,365,379) | 31.84 | — | 0.07 ± 0.01 | 0.27 ± 0.07 | 0.51 ± 0.08 | 0.32 ± 0.12 |
| Cholesterol | 32.43 | 59.74 ± 2.79 | 62.33 ± 1.68 | 66.61 ± 2.00 | 53.03 ± 2.51 | 74.51 ± 2.04 |
| Cholestanol | 32.47 | 1.40 ± 0.14 | 0.53 ± 0.08 | 0.90 ± 0.11 | 0.60 ± 0.06 | 0.55 ± 0.12 |
| Cholesta-5.7-dien-3-ol. | 32.65 | 13.41 ± 1.85 | 2.68 ± 0.54 | 8.02 ± 1.33 | 1.16 ± 0.19 | 0.54 ± 0.17 |
| Un.steroid (105,213,255,353,368,386,415) | 32.75 | 0.02 ± 0.01 | 0.03 ± 0.02 | 0.35 ± 0.11 | 0.09 ± 0.03 | 0.39 ± 0.16 |
| Ergosterol | 33.00 | — | 0.05 ± 0.02 | — | 0.17 ± 0.11 | — |
| Campesterol | 33.17 | 1.61 ± 0.22 | 3.76 ± 0.28 | 3.27 ± 0.36 | 5.46 ± 0.28 | 4.22 ± 0.57 |
| Cholest-4-en-3-one | 33.41 | 0.17 ± 0.03 | 0.53 ± 0.17 | 0.19 ± 0.05 | 0.20 ± 0.02 | 0.92 ± 0.38 |
| Ergosta-5,8-dien-3-ol | 33.50 | 2.43 ± 0.30 | 1.58 ± 0.22 | 2.38 ± 0.37 | 1.31 ± 0.24 | 0.56 ± 0.14 |
| Cholesta-4,6-dien-3-one | 33.69 | 0.24 ± 0.06 | 0.53 ± 0.08 | 0.29 ± 0.06 | 0.40 ± 0.06 | — |
| Sitosterol | 33.92 | 0.65 ± 0.10 | 0.74 ± 0.16 | 0.94 ± 0.15 | 1.18 ± 0.11 | 1.13 ± 0.23 |
| Ergostanol | 34.02 | 0.07 ± 0.01 | 0.08 ± 0.03 | 0.10 ± 0.02 | 0.11 ± 0.02 | 0.33 ± 0.11 |
| Stigmasterol | 34.13 | 0.31 ± 0.06 | 0.27 ± 0.13 | 0.28 ± 0.04 | 1.22 ± 0.22 | 0.44 ± 0.26 |
| Un.steroid (221,253,281,355,380,430) | 34.30 | 2.23 ± 0.32 | 0.70 ± 0.18 | 1.01 ± 0.16 | — | — |
| Cholest-5-en-3-one | 34.38 | — | — | — | 1.33 ± 0.24 | 0.91 ± 0.28 |
| Ergosta-5.22-dien-3-ol | 34.47 | — | 0.13 ± 0.07 | 0.12 ± 0.03 | 0.15 ± 0.04 | — |
| Un.steroid (214,267,395) | 35.30 | 0.12 ± 0.04 | 0.21 ± 0.11 | — | 0.56 ± 0.44 | 0.22 ± 0.09 |
| *Carboxylic acids and their esters* | | | | | | |
| Tetradecanoic acid | 20.64 | 0.16 ± 0.04 | 0.38 ± 0.13 | 0.22 ± 0.06 | 0.24 ± 0.05 | 0.85 ± 0.55 |
| Pentadecanoic acid | 21.68 | 0.13 ± 0.02 | 0.15 ± 0.12 | 0.10 ± 0.03 | 0.18 ± 0.05 | 0.41 ± 0.19 |
| Hexadecanoic acid. methyl ester | 22.33 | — | 0.05 ± 0.02 | — | 0.09 ± 0.02 | 0.25 ± 0.08 |
| Hexadecenoic acid | 22.54 | 0.16 ± 0.02 | 0.40 ± 0.20 | 0.25 ± 0.07 | 0.57 ± 0.33 | 0.28 ± 0.09 |
| Hexadecanoic acid | 22.76 | 3.68 ± 0.32 | 4.36 ± 0.65 | 3.11 ± 0.35 | 5.98 ± 0.51 | 1.54 ± 0.23 |
| Hexadecanoic acid, ethyl ester | 22.98 | — | 0.37 ± 0.11 | — | 0.19 ± 0.06 | 0.40 ± 0.17 |
| 9,12-octadecadienoic acid | 24.35 | 0.10 ± 0.01 | 0.11 ± 0.02 | 0.12 ± 0.02 | 0.27 ± 0.08 | 0.06 ± 0.02 |

Table 2: Continued.

| Compounds | RT (min) | Fuenfría | Pedrezuela | Golondrina | Belmonte | Aranjuez |
|---|---|---|---|---|---|---|
| Octadecenoic acid | 24.43 | 1.99 ± 0.18 | 1.76 ± 0.20 | 2.76 ± 0.57 | 4.82 ± 1.41 | 1.01 ± 0.21 |
| Octadecanoic acid | 24.60 | 1.39 ± 0.12 | 2.52 ± 0.34 | 1.41 ± 0.13 | 2.55 ± 0.23 | 0.99 ± 0.18 |
| Octadecanoic acid, ethyl ester | 24.82 | — | 0.51 ± 0.23 | — | 0.14 ± 0.04 | 0.55 ± 0.23 |
| Eicosanoic Acid | 26.31 | 0.46 ± 0.09 | 0.63 ± 0.15 | 0.76 ± 0.11 | 0.59 ± 0.17 | 0.64 ± 0.18 |
| Docosanoic acid | 28.00 | — | 0.01 ± 0.01 | — | 0.01 ± 0.01 | — |
| Docosanoic acid, ethyl ester | 28.21 | — | 0.45 ± 0.12 | — | 0.21 ± 0.05 | 0.23 ± 0.12 |
| *Alcohols* | | | | | | |
| Hexadecanol | 21.02 | 0.23 ± 0.05 | — | 0.19 ± 0.07 | 0.16 ± 0.04 | 0.16 ± 0.05 |
| Octadecanol | 23.87 | 0.26 ± 0.05 | 0.69 ± 0.16 | 0.19 ± 0.06 | 0.29 ± 0.08 | — |
| Eicosanol | 25.67 | 0.17 ± 0.03 | 0.55 ± 0.13 | 0.28 ± 0.08 | 0.21 ± 0.05 | 0.81 ± 0.28 |
| Docosanol | 27.33 | 0.23 ± 0.05 | 0.52 ± 0.15 | 0.23 ± 0.04 | 0.23 ± 0.04 | 0.73 ± 0.26 |
| Tetracosanol | 29.80 | 0.03 ± 0.01 | 0.07 ± 0.01 | 0.02 ± 0.01 | 0.07 ± 0.02 | 0.01 ± 0.01 |
| *Waxy esters* | | | | | | |
| Unidentified waxy ester 1 | 29.45 | 0.28 ± 0.08 | 0.98 ± 0.20 | — | 1.37 ± 0.47 | 0.75 ± 0.23 |
| Unidentified waxy ester 2 | 35.57 | 0.58 ± 0.10 | 2.61 ± 0.60 | 0.42 ± 0.08 | 2.84 ± 0.45 | 0.69 ± 0.26 |
| Unidentified waxy ester 3 | 38.06 | 0.23 ± 0.06 | 0.29 ± 0.06 | 0.20 ± 0.05 | 0.09 ± 0.03 | 0.37 ± 0.23 |
| Unidentified waxy ester 4 | 38.27 | 0.63 ± 0.11 | 1.78 ± 0.26 | 0.47 ± 0.10 | 2.26 ± 0.30 | 0.82 ± 0.16 |
| *Others* | | | | | | |
| Tetradecanone | 22.11 | 0.20 ± 0.05 | 0.27 ± 0.11 | 0.13 ± 0.03 | 0.15 ± 0.03 | 0.18 ± 0.06 |
| Unidentified Furanone | 24.19 | 0.12 ± 0.02 | 0.10 ± 0.03 | 0.06 ± 0.01 | — | — |
| Squalene | 30.07 | 0.93 ± 0.26 | 0.70 ± 0.10 | 0.66 ± 0.20 | 0.35 ± 0.04 | 0.40 ± 0.19 |
| Unidentified terpenoid 1 | 30.83 | 0.09 ± 0.03 | 0.07 ± 0.03 | 0.08 ± 0.02 | 0.03 ± 0.01 | 0.13 ± 0.07 |
| Unidentified terpenoid 2 | 31.94 | 0.48 ± 0.09 | — | 0.48 ± 0.12 | 0.05 ± 0.01 | — |

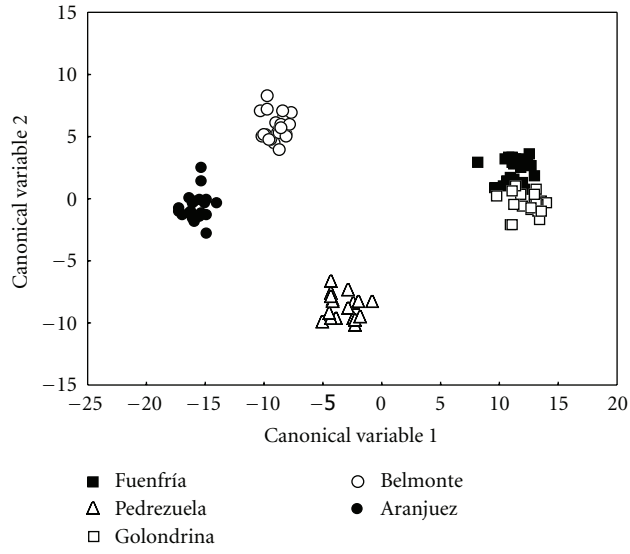


Figure 2: Separation of the principal components scores (PCs) describing chemicals from femoral secretions of male lizards in a discriminant analysis based on population of origin.

presented in all cases (Table 3; Figure 3). In all populations, males discriminated between scents of any male and water (Tukey's tests: $P < 0.005$ in all cases). Males from Aranjuez and Belmonte directed a significantly higher number of TFs to scent of males of their own population or of the other Southern population than to scent of males from the three Northern populations, which did not differ (Table 3; Figures 3(a) and 3(b)). The number of TFs directed by males from Fuenfría was significantly higher in response to scent of males of their own population than to scent of males from any other population, which did not significantly differ (Table 3; Figure 3(c)). Males from Golondrina directed a significantly higher number of TFs in response to scent of males of their own population than to males from Aranjuez, Belmonte and Pedrezuela (Table 3; Figure 3(d)). The number of TFs in response to scent of males of their own population and Fuenfría males was not significantly different, and the latter was not significantly different from the rest of populations. Finally, males from Pedrezuela directed significantly more TFs in response to males of their own population than to males of the two Southern populations (Aranjuez and Belmonte), which did not significantly differ (Table 3; Figure 3(e)). However, responses to males of their own population did not significantly differ from responses to males of the other two Northern populations.

*3.4. Chemosensory Responses of Males to Scent of Females.* The number of TFs differed between treatments in all populations (Table 3; Figure 4). In all cases, males discriminated between scents of any female and water (Tukey's tests: $P < 0.005$ in all cases). Males from Aranjuez and Belmonte directed a significantly higher number of TFs to scent of females of their own population than to scent of females from

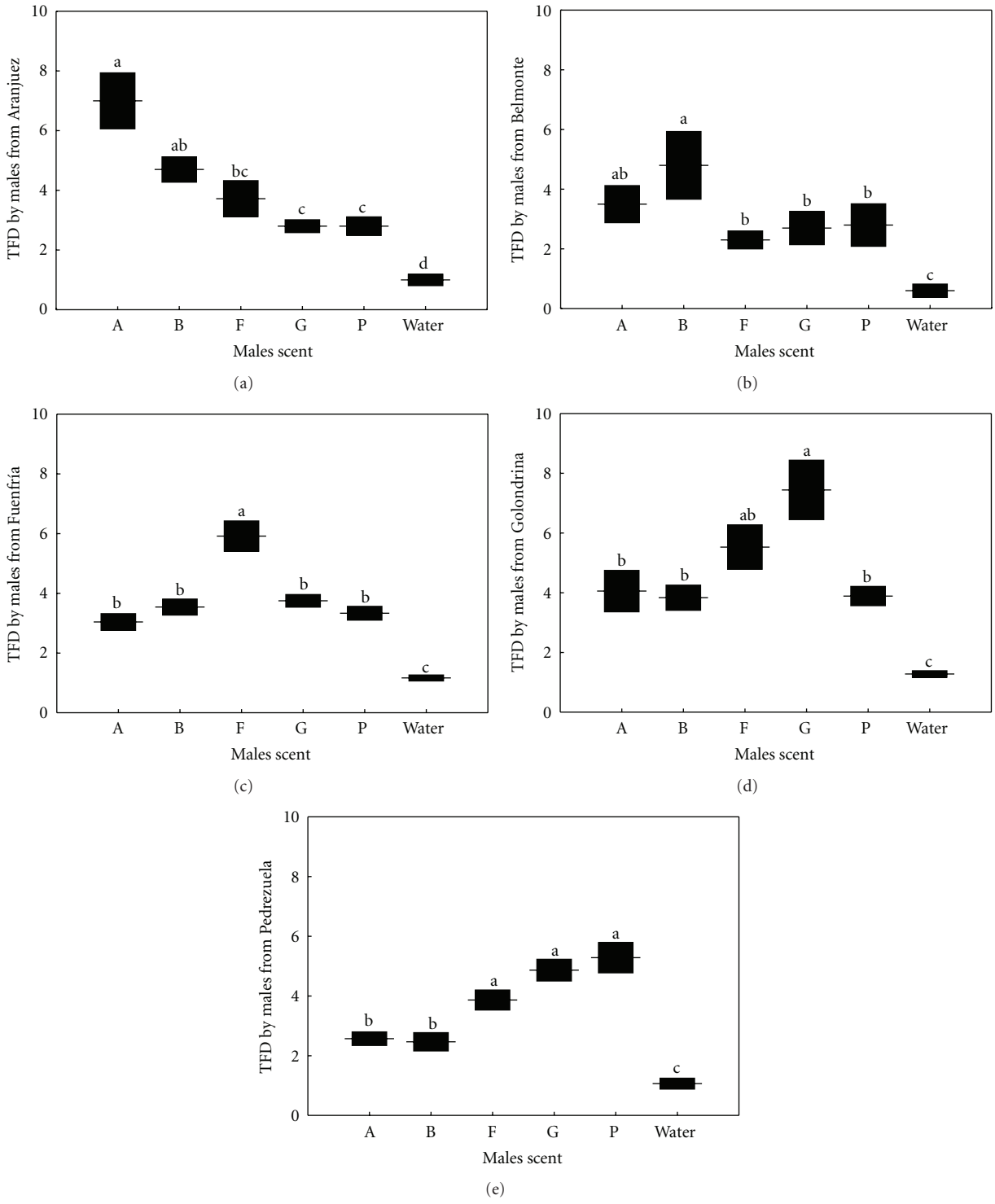


Figure 3: Tongue flicks directed (TFD; mean ± SE) by males from five populations of the Madrid region in response to swabs bearing scent of males of different populations (Aranjuez: A; Belmonte: B; Fuenfría: F; Golondrina: G; Pedrezuela: P) or a water odorless control. The same letter above the bars denotes a nonsignificant difference in post hoc tests.

all the Northern populations, which did not significantly differ (Table 3; Figures 4(a) and 4(b)). The number of TFs directed by males from Fuenfría was significantly higher in response to scent of females of their own population than to females from any other population (Table 3; Figure 4(c)). Males from Golondrina directed a significantly higher number of TFs in response to scent of females from the three Northern populations, including their own population, than

Table 3: Results from one-way repeated measures ANOVAs comparing the tongue flicks directed by individuals (males and females) from five distinct populations of the Madrid region (Aranjuez, Belmonte, Golondrina, Fuenfría, and Pedrezuela) in response to swabs bearing scent of males or females of the different populations or a water odorless control.

| | One-way repeated measures ANOVAs | | | | | |
|---|---|---|---|---|---|---|
| | *Responses of males to scent of males* | | *Responses of males to scent of females* | | *Responses of females to scent of males* | |
| | F | *P* | F | *P* | F | *P* |
| Aranjuez | 36.46 | <0.0001 | 24.39 | <0.0001 | 27.30 | <0.0001 |
| Belmonte | 35.98 | <0.0001 | 21.89 | <0.0001 | 33.67 | <0.0001 |
| Fuenfría | 54.42 | <0.0001 | 74.84 | <0.0001 | 46.84 | <0.0001 |
| Golondrina | 32.00 | <0.0001 | 28.00 | <0.0001 | 49.21 | <0.0001 |
| Pedrezuela | 39.19 | <0.0001 | 49.48 | <0.0001 | 33.12 | <0.0001 |

to females from the two Southern populations (Table 3; Figure 4(d)). Males from Pedrezuela directed significantly more TFs in response to scent of females of their own population than to scent of females from any other population (Table 3; Figure 4(e)). However, responses to scent of females from the two other Northern populations were significantly higher than to females from the two Southern populations, which did not differ.

*3.5. Chemosensory Responses of Females to Scent of Males.* The number of TFs differed between treatments in all populations (Table 3; Figure 5). All females discriminated between scents of any male and water (Tukey's tests: $P < 0.005$ in all cases). Females from Aranjuez and Belmonte directed a significantly higher number of TFs in response to scent of males of their own population than to males from the three Northern populations, which did not differ significantly (Table 3; Figures 5(a) and 5(b)). Females from Aranjuez and Belmonte did not significantly differ in their responses to scent of males of their own population or to males from the other Southern population (Belmonte or Aranjuez). The number of TFs directed by females from Fuenfría was significantly higher in response to scent of males of their own population than to males from the two Southern populations and from one of the Northern populations (Pedrezuela), which did not significantly differ (Table 3; Figure 5(c)). Responses to scent of males of their own population and to males from Golondrina were not significantly different, nor were different the responses to males from Golondrina and Pedrezuela. Females from Golondrina directed a significantly higher number of TFs in response to scent of males from the three Northern populations (Fuenfría, Pedrezuela, and their own population) than to scent of males from the Southern populations (Aranjuez and Belmonte) (Table 3; Figure 5(d)). Females from Pedrezuela directed significantly more TFs in response to scent of males from their own population than to males from all the other Southern and Northern populations, which did not differ significantly (Table 3; Figure 5(e)).

## 4. Discussion

Our study showed that different populations of Iberian wall lizards *P. hispanica* living within a relatively small geographical area, whose environmental conditions differed between population sites, differed in morphology and in the composition and proportion of chemical compounds in femoral gland secretions of males. Males of each population secreted a singular and characteristic mixture of compounds used as sexual signals. Tongue-flick tests showed that these differences resulted in differential chemosensory recognition between some populations. These results suggested that there could be premating reproductive isolation between some, but not all, populations of this lizard.

With respect to morphology, we could first differentiate between individuals from the South and North of the study area. Lizards from Fuenfría and Golondrina (i.e., Northern populations) were characterized by being larger, heavier, and with larger, more robust heads than individuals from Aranjuez and Belmonte (i.e., Southern populations). These differences could be explained by the different contrasting environments where these populations live, Northern mountains (with cold temperature, high humidity, and high altitude) versus Southern plains (hot temperatures, dry conditions, and low altitude). Variations of body size of many animals, and in particular of vertebrates, are often explained by phenotypic plasticity or local adaptation to different climatic conditions, with individuals from colder environments being larger than those from warmer areas (e.g., [51]). Lizards with a large body size have low thermal inertia (i.e., low cooling rates) [52], and this may be an adaptation to increase effectiveness of thermoregulation in the Northern populations where ambient temperatures are relatively cold, in contrast to the Southern populations where temperatures are warmer and lizards are smaller.

Moreover, Iberian wall lizard populations differ in the number of femoral pores and blue spots, with males from the Northern populations having more femoral pores and less blue spots than males from the Southern populations. Only lizards from the Pedrezuela Northern population had an intermediate number of femoral pores. Because femoral pores and blue spots are used in chemical and visual intraspecific communication, respectively (e.g., [37, 39]), it is likely that the importance of these two sensory modes differ between populations. A higher number of femoral pores may be related to a higher production of chemical secretions [53], whereas a larger number of blue spots may represent a higher use of visual signals [45]. The relative importance of chemical and visual signals may be explained by the effectiveness of

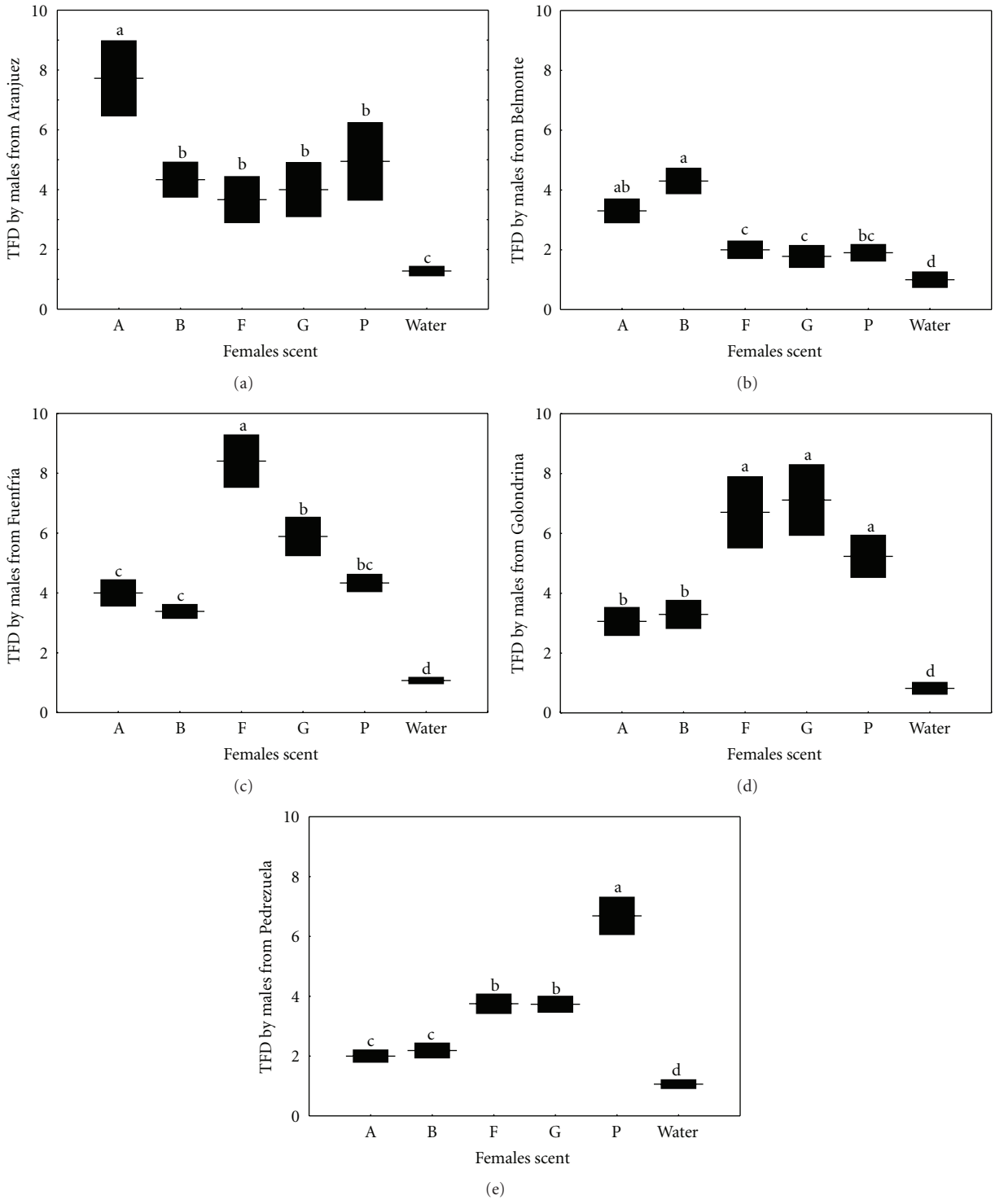


Figure 4: Tongue flicks directed (TFD; mean ± SE) by males from five populations of the Madrid region in response to swabs bearing scent of females of different populations (Aranjuez: A; Belmonte: B; Fuenfría: F; Golondrina: G; Pedrezuela: P) or a water odorless control. The same letter above the bars denotes a nonsignificant difference in post hoc tests.

these two types of communication in different environments [4, 54], which might have affected the evolution of sexual signals of different populations of *P. hispanica* lizards.

In fact, the chemical analyses showed that, similarly to other lizard species, femoral gland secretions of *P. hispanica* have carboxylic acids and steroids as predominant components (reviewed in [55]). However, compounds found in femoral gland secretions of male *P. hispanica* varied in composition and proportions between populations, and these variations alone would allow a characterization of males

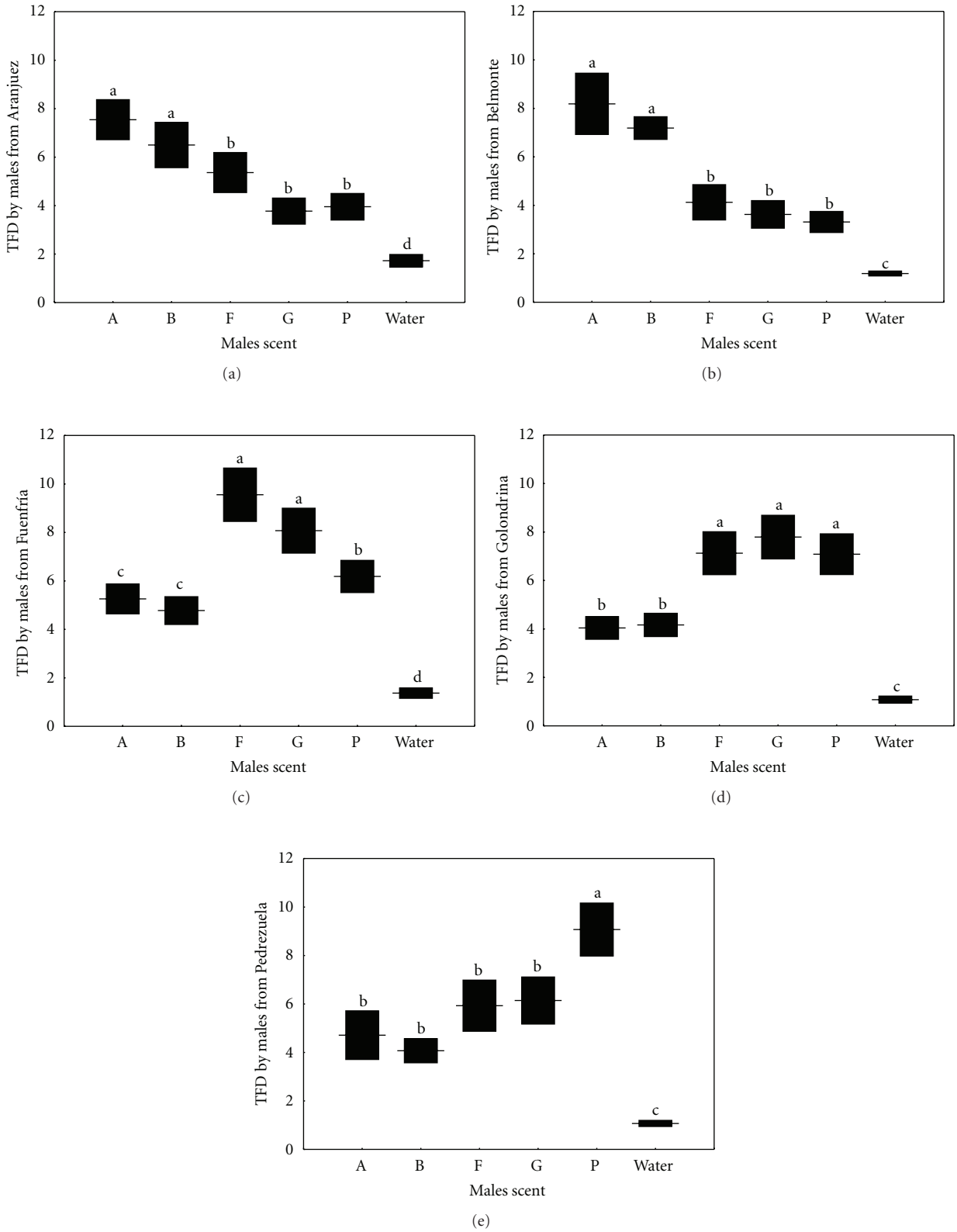


Figure 5: Tongue flicks directed (TFD; mean ± SE) by females from five populations of the Madrid region in response to swabs bearing scent of males of different populations (Aranjuez: A; Belmonte: B; Fuenfría: F; Golondrina: G; Pedrezuela: P) or a water odorless control. The same letter above the bars denotes a nonsignificant difference in post hoc tests.

from each population. These differences could be due to local adaptation to the habitats of each population [4, 54]. Selection for a better efficiency of substrate scent marks might have led to differences in composition of secretions of lizards inhabiting distinct environments, with less volatile and stable molecules being found in the Southern populations where temperature and evaporation rates were higher [4, 11]. Also, differences in secretions might be related to different diets or differently available food sources [5]. The question that arises is whether these differences in chemicals affect recognition systems and whether this may have consequences for speciation.

Chemosensory recognition experiments showed that individuals of *P. hispanica* from each population could clearly detect scents of lizards from any population in comparison with an odorless control (i.e., water). However, lizards showed different tongue-flick (TF) rates depending on the population of origin of the lizard's scent presented. Both females and males varied in their responses to scents from lizards from the different populations. Males showed more "interest" (i.e., a higher TF rate) for scents from males from their own area (i.e., North versus South); males from the Northern populations made more TFs in response to scent of males from the Northern populations than to scent of Southern males. Similarly, Southern males made more TFs in response to scents from Southern males than to scent from Northern males. Only males from Fuenfría population showed a clearly higher response to scents from males of their own population. For the rest of populations, there were not higher responses to scent of males from their own population, but there was a recognition of the area of origin (North versus South) of the male.

Moreover, males also discriminated between scents from females from the different areas. Males from Northern populations showed more interest for scents from Northern females than for scents from Southern females; similarly this occurred in Southern males. However, we observed one interesting difference: males from the populations that are geographically located far away from the others (i.e., Aranjuez, Fuenfría, and Pedrezuela) showed a clear discrimination and interest (i.e., higher TF rates) for scents of females from their own population against scent of females from any other population. There was also a further secondary intermediate interest for scent of females from other populations of their own area and finally a lower interest for females from the other area. In contrast, for the populations geographically located in the middle of the Madrid region (i.e., Belmonte and Golondrina), we did not observe a discrimination nor a higher interest of males for scent of females from their own population, but only a discrimination of females from their own area.

In addition, we found similar results for the males' scents recognition by females. Females recognized the area of origin of the male (South versus North). Females from Northern populations made more TFs in response to scent of Northern males than to Southern males, and vice versa, but there were no differences between populations within each area. We found only a clearly higher interest of Pedrezuela females for scents of males from their own population against all the other populations.

These results seem concordant with the previous description of morphotypes of *P. hispanica* using morphological and genetic data [29, 32–34]. Thus, Northern populations would be close to those described for the morphotype 1, while the Southern populations would be more similar to the morphotype 2. However, we observed a particular result for lizards from Pedrezuela population; these lizards live in the North, but they have chemical and morphological differences with respect to other Northern populations. Lizards from Pedrezuela have a morphology intermediate between Southern and Northern populations. Moreover, the chemical signals in this population are singular in comparison to the other populations, and this chemical signature is effective in the chemosensory recognition of scent of males and female from their own population. Therefore, the assignation of this population to previously described morphotypes is not clear.

In summary, our results showed that male and female *P. hispanica* lizards from five distinct populations of the Madrid region can recognize and discriminate between scents of individuals from the Northern and Southern populations, and have more interest for scents of lizards from their own area than for scents of lizards from the other area. Moreover, males from some populations discriminate and maybe prefer scents of females from their own population than from any other populations. This clear ability of males to discriminate between some female populations might suggest that there is a cryptic speciation process, probably mediated by the role of chemical signals in sexual interactions. However, we need further mating experiments to test this. In addition, females also seem to discriminate male chemicals between areas (North versus South), but not between populations. All these results support that reproductive isolation between all the distinct populations of *P. hispanica* is not entirely clear, but that, at least between some populations, there could be reproductive isolation and cryptic speciation, which merits further studies.

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*Review Article*

# 170 Years of "Lock-and-Key": Genital Morphology and Reproductive Isolation

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The divergent genital morphology observed among closely related animal species has long been posited as a mechanism of reproductive isolation. Despite the intuitive appeal that rapidly evolving genitalia might cause speciation, evidence for its importance—or even its potential—in reproductive isolation is mixed. Most tests of genital structural isolation between species often fail to find convincing evidence that differences in morphology prevent copulation or insemination between species. However, recent work suggests that differences in genital morphology might contribute to reproductive isolation in less obvious ways through interactions with sensory mechanisms that result in lowered reproductive fitness in heterospecific matings. In this paper, I present a brief history of the "lock-and-key" hypothesis, summarize the evidence for the involvement of genital morphology in different mechanisms of reproductive isolation, discuss progress in identifying the molecular and genetic bases of species differences in genital morphology, and discuss prospects for future work on the role of genitalia in speciation.

*L'armure copulatrice est un organe ou mieux un instrument ingénieusement compliqué, destiné à s'adapter aux parties sexuelles externes de la femelle pour l'accomplissement de l'acte copulatif; elle est la garantie de la conservation des types, la sauvegarde de la légitimité de l'espèce.*
[The copulation armor is an organ or better an instrument ingeniously complicated, destined to adapt to sexual parts external to the female for the completion of copulation; it is the guarantee of the preservation of the standards, the safeguard of the legitimacy of the species.]
L. Dufour, 1844

## 1. Introduction

The French entomologist Leon Dufour's statement [1] in which he hypothesized that the remarkable diversity in genital morphology observed among Dipterans is important for maintaining reproductive isolation (RI) between species is one of the notable ideas in speciation research that has generated both considerable controversy and much experimental work. Indeed, among different mechanisms of RI, structural isolation involving genitalia appears to be one of the first mechanisms of speciation to be experimentally tested. However, despite the widespread differences in genital morphology observed among many animal species [2], and the intuitive nature of the so-called lock-and-key hypothesis—that structural differences in the genitalia prevent species from hybridizing—the vast majority of experimental tests have failed to find convincing evidence that the differences in genitalia between species have a substantial role in preventing hybridization. Most of these tests, however, have focused on one specific mechanism of genital lock-and-key RI, and recent studies suggest that differences in genital morphology might in fact contribute to RI in more cryptic ways that reduce the reproductive success of heterospecific matings.

Lock-and-key reproductive isolation can operate via two different mechanisms [3, 4]. The first is the classic mechanical or structural lock-and-key mechanism of Dufour where differences in genital morphology between species prevent or reduce the success of copulation and/or insemination as a direct result of mechanical incompatibilities that occur

during genital coupling. RI caused by structural lock-and-key also includes postmating fitness losses such as physical damage to either parent that might prevent any future matings. The second mechanism is sensory lock-and-key first posited by De Wilde [5] and later formalized by Eberhard [3] where differences in genital morphology are perceived by one or both sexes and evoke behavioral or physiological responses that result in premature termination of mating attempts or postcopulatory reproductive fitness problems. These two mechanisms are not mutually exclusive, and both can operate in concert to give rise to RI.

Because genital morphology and its role in RI have received much attention in literature, it will be helpful to make clear what I will discuss in this review. Several hypotheses about the forces that drive the rapid evolution of genital morphology in animal taxa have been put forth, and there is good evidence that sexual selection often operates to drive the evolution of genital morphology within species. These hypotheses and the data supporting them have been reviewed thoroughly elsewhere [2, 6–8], and I refrain from doing so here. I also refrain from reviewing the evidence testing genital lock-and-key RI using comparative or phylogeographic data. My reason for doing this is that without detailed knowledge of the evolutionary histories of species, it is often difficult to draw firm conclusions about the role of genitalia in RI from these patterns alone. For example, although patterns like reproductive character displacement are consistent with reinforcement acting on genital morphology where species exist either sympatrically or parapatrically, an absence of reproductive character displacement does not preclude a history of differences in genital morphology contributing to RI between species, as other RI mechanisms (e.g., mate discrimination, ecological niche divergence, temporal isolation, etc.) might evolve via reinforcement before reproductive character displacement might evolve.

Instead, my goal here is to examine evidence of hybridizations between species where differences in genital morphology appear to contribute to RI either by structural lock-and-key and/or sensory lock-and-key mechanisms. Although many studies exist that present indirect evidence to suggest a possible role for genitalia in contributing to RI between species, I focus my discussion on only those studies that have either directly observed species crosses in nature, or performed laboratory crosses between species, and recorded postcopulatory reproductive fitness in heterospecific pairs. In each of these cases it is important to emphasize that other mechanisms of RI beside genital lock-and-key also exist between these species pairs, and I do not suggest that genital isolation is the primary cause of speciation in any of these examples. Rather, I present these data to illustrate the possibility that RI between species can occur as a byproduct of differences in genital morphologies that have evolved in response to evolutionary forces such as sexual selection acting within species. In describing each of the heterospecific crosses below, I use the convention of always presenting the female parental species first (e.g., *species* A female × *species* B male).

## 2. The History of the Idea

Observations of genitalia and structural isolation have enjoyed a long history in the study of speciation and systematics (for a detailed history of the structural lock-and-key hypothesis and tests of the hypothesis, see [9]). Much of the appeal of the structural lock-and-key hypothesis is that it offers an intuitively obvious mechanism of RI and an appealing visual image. The idea that incompatibilities between genitalia of different species caused RI was perhaps considered so obvious that it appears few careful tests of structural isolation were performed in the years following Dufour. Among some of the early proponents of the idea was one of Dufour's students, Pérez [10], who made similar observations that genital morphology among several Hymenoptera species was incredibly diverse, and supported the notion that these differences in male genital morphology were the likely cause of RI among species (Although Pérez seemed to accept that differences genital morphology are important for RI, he does urge some caution in his interpretation as he notes that in some groups the genital morphology among males varies very little. It is a bit unclear, however, if his caution is in regard to the potential for genitalia to cause RI, or for their usefulness in systematic classification.). Another early champion of the idea, and one of the first to make careful observations of genital coupling between males and females in a laboratory setting, was Jordan [11]. Jordan performed crosses between species of *Papilio* and observed that for crosses within species, the male and female external genitalia are well-matched and fit together with tight coupling, but in crosses between species, the coupling between the male and female genitalia is not quite as good. Specifically, Jordan found that although the external genitalia of *Papilio memnon* males and females fit to provide tight genital coupling, in crosses between *P. helenus* and *P. memnon* and between *P. podalirius* (the modern classification of this species is *Iphiclides podalirius*) and *P. machaon*, the male and female genitalia did not fit as precisely to secure the copulating pairs as they did within species. Jordan also observed that, similar to males, the females of different species also possess genital structures that appear different, but these differences are not as dramatic as those observed among male genital structures.

Despite these early observations that seemed to support structural lock-and-key RI, many subsequent observations called the ubiquity, and even the existence, of RI as a result of differences in genital morphology into question. Boulangé [12] pointed out that in many Hymenoptera species, the most divergent structures of the male genitalia are those that are *not* involved in intromission and insemination. Instead, these structures make contact with female structures that possess essentially identical morphology across species, for example, the sides of the female abdomen. He also observed that in comparisons between species where females do possess divergent genital morphology, the differences are usually minor. Richards [13] and later Robson and Richards [14] made several observations in *Bombus* (subgenus *P sithyrus*) that also did not support the structural lock-and-key hypothesis. Among the many criticisms they level against structural lock-and-key, they found that females usually do not differ

greatly in genital morphology, so the required structural "locks" simply do not exist, the morphological differences in male genitalia of some species are so small as to likely have no effect on reducing the tight coupling between male and female genitalia, and there appears to be no correlation between the degree of diversity in genital morphology among species and the species richness of most groups. Robson and Richards also presented what is perhaps the most serious criticism against the importance of structural lock-and-key in RI: species that possess dramatic differences in genital morphology can often mate and produce offspring. From these observations, they state with regard to RI, "we are forced to regard specific differences in the genitalia as of essentially the same nature as other apparently useless specific characters" [14, page 297]. They conclude that divergent genital morphologies are more likely the result of RI rather than its cause, and other isolating mechanisms (in particular mate discrimination) probably play more important roles in speciation. For many years following, tests for genital structural isolation were performed in a variety of animal taxa and almost all of these tests failed to find convincing evidence of structural lock-and-key RI [9].

What exactly "lock-and-key RI" implies has also become a bit muddled throughout the literature since the introduction of the idea. A common notion of lock-and-key RI describes the phenomenon as natural selection acting on genital morphology *to prevent species from hybridizing*. When considering the case of reinforcement, this is certainly an applicable definition, but as a more general definition to describe genital lock-and-key RI, it is incorrect. Just as some other RI mechanisms such as intrinsic postzygotic isolation evolve as a byproduct of evolutionary processes that occur within populations evolving in isolation, genital lock-and-key RI can also evolve as a byproduct of evolutionary processes that occur within isolated populations, such as sexual selection, that can act to drive genital morphological evolution. The divergence in morphology between two populations might consequently give rise to either structural or sensory isolation as a byproduct when they attempt to hybridize. A history of sympatry or parapatry is not required for lock-and-key RI to evolve under this scenario, and I use this broader definition of lock-and-key in evaluating the contribution of genitalia to RI.

## 3. Structural Isolation

For structural isolation to operate two criteria need to be satisfied. First, the genitalia of both males and females are required to bear substantial, species-specific differences in morphology of structures important for successful copulation, intromission, and/or insemination. Second, male and female genitalia *within* species are required to show strong correlated evolution in the morphology of these structures. Tests of structural isolation are often difficult as they require both incomplete premating RI between species and careful measurements of parental fitness loss after heterospecific matings. Despite these challenges, crosses between species in a handful of arthropod and arachnid taxa suggests that structural lock-and-key may, in fact, contribute to RI in at least some hybridizations.

Standfuss [15, pages 60-61] observed crosses between 24 heterospecific pairs of the lackey moths *Bombyx franconica* and *B. neustria* (modern day classifications for these two species are *Malacosoma franconica* and *M. neustria*, resp.). This large number of heterospecific matings suggests that little premating isolation exists between these two species, and indeed, courtship and copulation appear to proceed normally [15]. After mating, the *M. franconica* females also appear to exhibit the normal postcopulatory behavior by walking around to identify locations to oviposit. Within the next three to four hours of observation, however, most of these females die. Among those that survive, some are unable to lay eggs. Others do lay eggs, but egg lethality ranges from 50–100% and larvae never reach adulthood. Although no description is given of the morphological differences in male or female genitalia between these two species, Standfuss speculates that the probable cause of lethality of *M. franconica* females was injuries suffered as a result of the *M. neustria* male genitalia. He is, however, cautious in his interpretation, and hypothesizes that other species differences might have been the cause of female lethality (In a remarkably insightful passage for the time period, Standfuss hypothesizes that some of the other possible causes of egg mortality and female lethality might include sperm-egg incompatibility and what he describes as "molecular differences" between the two species.).

Structural isolation also appears to operate in some other species of Lepidoptera. Federley [16, pages 371-372] performed crosses between the hawk moths *Metropsilus porcellus* and *Chaerocampa elpenor* (modern day classifications for these two species are *Deilephia porcellus* and *D. elpenor*, resp.). Upon intromission, *D. elpenor* males become "stuck" and are unable to withdraw their aedeagus from the *D. porcellus* females. In the case of copulating species pairs that do manage to separate, females never lay eggs, although sperm is found in the bursa copulatrix, which suggests the possibility that damage to the female reproductive tract might prevent fertilization and/or oviposition. Males of these two species bear substantial morphological differences in their external genitalia: *D. elpenor* males possess a long and slender aedeagus, whereas *D. porcellus* males possess a shorter and thicker aedeagus compared to those of *D. elpenor* males. These differences in male genitalia are consistent with structural isolation in the *D. porcellus* × *D. elpenor* cross, and also agree with the results from the reciprocal cross—*D. elpenor* × *D. porcellus* proceeds normally through intromission and insemination [16].

Males and females of several species of crab spider in the genus *Misumenops* possess genital morphologies that are both highly divergent among species and show strong correlated evolution between males and females within species [17]. During copulation, the male intromittant organ, the palpus, is guided into the reproductive tract by the female epigynum to successfully complete intromission and insemination. In the cross *M. rothi* × *M. gabrielensis*, *M. gabrielensis* males initiate courtship and are received by the female, but are unable to orient their palpus and complete intromission

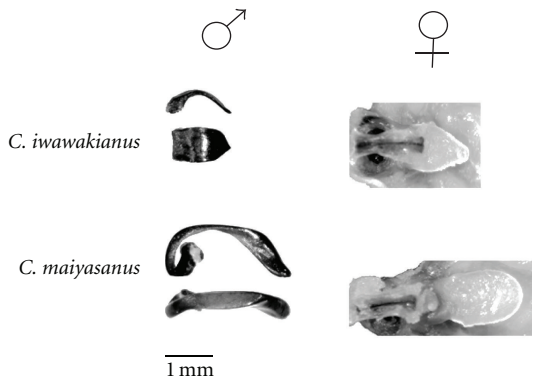


Figure 1: Genital morphology in *Carabus iwawakianus* and *C. maiyasanus*. The male copulatory piece is shown from both dorsal and sagittal views. The female vaginal appendix for each species is also shown. Images courtesy of Teiji Sota.

because the *M. gabrielensis* palpus is much larger than the opening to the epigynum in *M. rothi*. (It seems possible that the reciprocal cross might proceed through intromission and insemination, but was not attempted [17].) In the cross *M. lepidus* × *M. californicus* attempts at intromission were unsuccessful because the *M. californicus* male failed to correctly align the palpus with the epigynal structures of the *M. lepidus* female presumably because of the structural incompatibilities between the two structures. The reciprocal cross results in successful intromission and insemination, but does not produce offspring. Individual females have been collected from nature that show genital morphology intermediate to those of *M. lepidus* and *M. californicus*, thus it does appear that the structural RI in the *M. lepidus* × *M. californicus* cross is incomplete [17].

One of the best-characterized examples of structural lock-and-key comes from hybridizations among beetles of the genus *Carabus* (subgenus *Ohomopterus*) [18]. *Carabus* comprises a large group of wingless beetles on Japanese island of Honshu. Many species in the genus exist parapatrically and form hybrid zones. In the *C. iwawakianus* and *C. maiyasanus* species pair, males of both species do not discriminate against heterospecific females, and hybrid individuals that possess intermediate genital morphology between these two species have been found at low frequencies in the hybrid zone. Males of each species possess striking differences in genital morphology: *C. iwawakianus* possesses a short and wide copulatory piece, whereas *C. maiysanus* possesses a long and thin copulatory piece (Figure 1). The vaginal appendix of the females in each species also shows striking correlated morphology with the copulatory piece of their conspecific males (Figure 1). During intromission, the copulatory piece of the male and vaginal appendix female lock together. Sota and Kubota [18] performed reciprocal crosses between these two species and measured male and female fitness after intromission. In the cross *C. iwawakianus* × *C. maiyasanus* 50% (18 of 36) of *C. maiyasanus* males suffer broken copulatory pieces that were likely to prevent future matings. Female *C. iwawakianus* mortality is high (60%, 20 days postmating) and postmortem dissections revealed ruptured vaginal appendices and torn bursae in the majority of cases. In the reciprocal cross *C. maiyasanus* × *C. iwawakianus*, none of the *C. iwawakianus* males suffer injuries to the copulatory piece (0 of 27), and female *C. maiyasanus* mortality is lower compared to the *C. iwawakianus* × *C. maiyasanus* cross (∼30% versus 60%). However, a substantial fraction of *C. maiyasanus* females still suffer damage to the vaginal appendix that appear severe enough to prevent future mating. In both directions of crosses between these species, mated females lay fewer eggs and the egg hatch rates are lower, although it is unclear whether this is a direct consequence of damage to the female reproductive tract by the male genitalia. Structural isolation might prove to be a common mechanism of RI among *Carabus* species as female mortality following heterospecific crosses has also been reported between *C. albrechti* and *C. iwawakianus* [19] and between *C. albrechti* and *C. yamato* [20].

Another well-characterized example of a species group where structural lock-and-key appears to have an important role in RI occurs among some species of millipedes [21]. The *Parafontaria tonominea* species complex are also endemic to Japan, and many species exist parapatrically (some species exist sympatrically). Tanabe and Sota [21] performed crosses between *Parafontaria tonominea* sp. A and *Parafontaria tonominea* sp. B., two species that differ in their overall genital morphology, particularly in the size of their genital structures. They also possess notable differences in body size with *Parafontaria* sp. A possessing a larger body size than *Parafontaria* sp. B. Courtship and intromission between these species requires multiple steps and is highly choreographed. Courtship is initiated by the male with antennal contact, the male aligns head-to-head with female, and a preliminary intromission occurs without sperm transfer. If the female remains receptive following preliminary intromission, true intromission and insemination occur, which is then followed by postcopulatory behavior by the male. When preliminary intromission fails the male aborts the mating attempt. No apparent premating isolation exists between *Parafontaria* sp. A and *Parafontaria* sp. B, as males are equally likely to mate with a female of either species [21].

In the cross *Parafontaria* sp. B × *Parafontaria* sp. A, preliminary intromission fails in every cross (10 of 10) despite repeated contact between the male and female genitalia. The cause of the failed intromission is the difference in size between the much larger *Parafontaria* sp. A male gonopod and the smaller *Parafontaria* sp. B female gonopore. In the reciprocal cross *Parafontaria* sp. A × *Parafontaria* sp. B, preliminary intromission fails in ∼65% of the attempts (9 of 14) because the smaller *Parafontaria* sp. B male is unable to align his gonopod correctly even after repeated attempts due to his smaller overall body size. Four of the five remaining *Parafontaria* sp. A × *Parafontaria* sp. B crosses resulted in successful preliminary intromission, and true intromission occurred in three of the five. In each of these cases, however, the *Parafontaria* sp. B male terminated postcopulatory behavior prematurely.

A recent study has also identified structural isolation between two members of the *Drosophila melanogaster* species subgroup. *D. yakuba* and *D. santomea* inhabit the island of

Saõ Tomé off the western coast of Africa. Although these two species occur primarily at different elevations, their ranges overlap and form a hybrid zone at mid-elevation locations around the island [22, 23]. *D. yakuba* males possess a pair of sclerotized spikes that project from the lateral portion of the aedeagus. *D. santomea* males also possess sclerotized projections in the same location, but the morphology of the projections is rounded and more "nub-like." Females of each of these species show correlated evolution of genital structures with those of their conspecific males: *D. yakuba* females possess a pair of heavily sclerotized cavities that receive the male aedeagus spikes during copulation, whereas *D. santomea* females lack these cavities.

Kamimura and Mitsumoto [24] studied the consequences of these morphological differences on structural isolation between *D. santomea* and *D. yakuba*. In the cross *D. santomea* × *D. yakuba*, *D. yakuba* males successfully mount the *D. santomea* females, but usually fail to insert their aedeagus during mating, which results in insemination of only 20% (3 of 15) of the females in heterospecific matings. In 11 of the 12 remaining crosses that did not result in insemination, the male ejaculate was observed as a white, sperm-bearing mass on the external genitalia of either the *D. yakuba* male or *D. santomea* female in each pair. This mass appears to bind the genitalia of mating pairs together as heterospecific pairs are often observed struggling to separate from each other. Moreover, severe copulatory injuries were observed in mated *D. santomea* females that match the pattern of aedeagus spikes from the *D. yakuba* male. The reciprocal cross *D. yakuba* × *D. santomea* proceeds through copulation and insemination, although it is easy to dislodge mating pairs, which suggests that the spikes in *D. yakuba* may have evolved to secure genital coupling [24]. Similar modifications of male genitalia that function to secure genitalia during copulation within species have also been documented in *D. bipectinata* [25].

## 4. Sensory Isolation

*It has been thought that the highly specific shape of structures and appendages of male and female copulatory apparatus constitutes a decisive structural factor in species isolation, acting as a system of key and lock. But it would rather seem that intraspecific matings are assured by precopulatory behavior, and probably by the mutual stimulation of specific sensory sites during the copulatory act.*

J. De Wilde, 1964

The possibility that genital incompatibilities might cause RI between species through behavioral or physiological responses has recently received renewed interest. In contrast to the requirements for structural lock-and-key RI where both male and female genital structures are required to possess both complementary morphologies within species and divergent morphologies between species, sensory lock-and-key RI requires that only *one* of the sexes possesses divergent morphology between species. The morphology of the opposite sex, whether species-specific or not, requires innervation with sensory neurons capable of relaying information about species identity. Compared to structural lock-and-key mechanisms, sensory lock-and-key is perhaps more difficult to detect for the primary reason that the phenotype being studied is more complex—rather than studying a simple structural incompatibility, it is necessary to study a structurally induced behavior, or structurally induced changes in physiology. Although RI via sensory lock-and-key has not been as extensively studied as structural lock-and-key, there is growing evidence that it operates in some species crosses.

In laboratory crosses among several genera of Lepidoptera, forced matings often show no evidence of structural lock-and-key preventing successful insemination in heterospecific matings, despite that fact that male genital morphology often differs dramatically among closely related species (e.g., [26]). Although differences in male genital morphology do not appear to hinder the mechanics of copulation and intromission, they may relay species identity between the sexes. Two species of brush-footed butterflies *Erebia nivalis* and *E. cassioides* form hybrid zones along altitudinal gradients at some locations in the Alps. Although strong mating discrimination exists between them, heterospecific crosses can be obtained in the laboratory [26]. In conspecific crosses, mating pairs will remain in copula ∼18–30 minutes after a male successfully courts a female. However, in the heterospecific cross *E. nivalis* × *E. cassioides*, the male terminates copulation after only 0.5–7 minutes [27]. During this time the female remains receptive to the heterospecific male, and presumably the male perceives the female is heterospecific and terminates copulation prematurely, although it is also possible that the female fails to cooperate with the male and as a result he terminates the mating attempt. The duration of copulation in the reciprocal cross *E. cassioides* × *E. nivalis* appears normal [27].

Another example sensory lock-and-key occurs among some scarab beetle species [3]. *Macrodactylus costulatus*, *M. sylphis*, and *M. sericinus* females each possess relatively similar genital morphologies, but males of these species possess substantial differences in their genitalia. The differences in male morphology are most pronounced not in the hard cuticular structures of the external genitalia, but rather in the soft tissues involved in intromission. Among these three species, Eberhard [3] observed 37 male mounting attempts between heterospecific pairs out of total 160 mounting attempts in the field. All attempts to mount by heterospecific males were short (<5 seconds) and intromission never occurred. *Macrodactylus* females are known to reject unwanted males in conspecific crosses by contracting their vaginal walls to prevent intromission [28], thus it seems likely that females perceive heterospecific males and reject their copulation attempts using a similar behavior. However, it also seems possible that because the male morphological differences occur in soft and possibly sensory tissues, the male might perceive species identity of females via these differences in genital morphology, and terminate mating attempts prematurely.

Damselflies present an interesting case where both structural and sensory lock-and-key mechanisms appear to

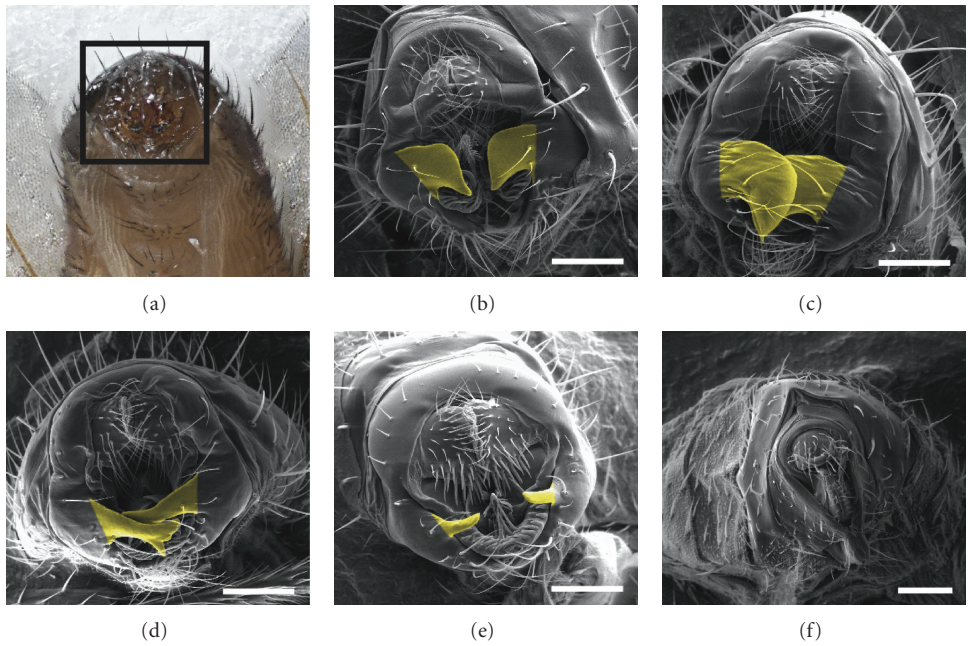


Figure 2: External genital morphology among members of the *Drosophila melanogaster* species complex. (a) Terminal portion of the male abdomen representative of the four members of the *D. melanogaster* species complex. Black box denotes the area of male genitalia shown in the scanning electron micrographs presented in panels (b–e). (b) *D. melanogaster* male, (c) *D. simulans* male, (d) *D. sechellia* male, (e) *D. mauritiana* male. Yellow shading marks the posterior lobe of the genital arch. (f) Female genital morphology representative of members of the *D. melanogaster* species complex. Scale bars are 100 $\mu$m.

contribute to RI. During courtship, the male damselfly grasps the female with his legs and brings his terminal appendages into contact with two mesostigmal grooves on the back of the female thorax. Both the male appendages and the female mesostigmal grooves within species show structural complementary and species-specific morphologies [29, 30]. Ablation experiments show that the male terminal appendages are important for mate recognition and copulation; females perceive the superior appendages, whereas the inferior appendages are used primarily by males for grasping the female [30]. Unreceptive females resist copulation by vigorously beating their wings and orienting their abdomen to prevent the males from achieving genital coupling. If the female is receptive, she bends her abdomen to make contact between her genitalia and those of the male. When damselflies occur in large groups, males will usually mate with conspecific females, but heterospecific matings do occur in nature [31].

In several genera of damselflies, the fit between the male appendages and the female mesostigmal grooves appears to be used by the female to assess species identity of the male. In heterospecific crosses among different *Sympecma* and *Lestes* species, heterospecific females vigorously resist copulation shortly after the male grasps the female and makes contact with his terminal appendages [31]. Similar observations have also been made in crosses between *Ischnura elegans* males mated to females of species belonging to different damselfly genera [32]. Paulson [33] performed several laboratory crosses among *Enallagma, Argia, Ischnura*, and *Telebasis* species. He observed that in contrast to conspecific pairs, in heterospecific pairs, the male appendages are unable to grasp the female thorax correctly. Roughly 66% of the crosses (8 of 12) show nearly complete inability of grasping by heterospecific males, which suggests structural incompatibilities between the morphologies of the male appendages and the female mesostigmal grooves also contribute along with sensory isolation to RI among these species.

Evidence of sensory lock-and-key is also found in some *Drosophila* species. Among members of the *D. melanogaster* species complex (*D. mauritiana, D. melanogaster, D. sechellia, D. simulans*), males possess two bilaterally symmetrical sclerotized cuticular genital structures called the posterior lobes of the genital arch, which insert between the eight and ninth tergites of the female during copulation [34], and differ dramatically in both size and shape among species (Figure 2; [35, 36]). Females of these species show no apparent differences in external genital morphology, although the posterior lobe likely comes into contact with, or distends soft abdominal tissues during copulation. No evidence exists of a structural lock-and-key mechanism among the *D. melanogaster* complex species that involves the posterior lobe, but the posterior lobe does appear important for mounting and genital coupling [37, 38]. (It is worth pointing out, however, that the posterior lobe in *D. sechellia* and *D. simulans* is known to cause some damage to the soft abdominal tissues at the insertion site in conspecific crosses [39].)

Heterospecific crosses among *D. mauritiana, D. sechellia,* and *D. simulans* show two cryptic defects that reduce the reproductive success of heterospecific pairs. The first defect is that copulation is generally shorter when females mate with heterospecific males compared to copulation duration

when females mate with conspecific males. Within species, copulation duration lasts an average of ~15–17 minutes in *D. mauritiana*, ~30 minutes in *D. sechellia*, and ~25–30 minutes in *D. simulans* [37, 40, 41]. In the *D. simulans* × *D. mauritiana* cross, copulation is much shorter than either pure species cross and lasts only 5–11 minutes [37, 40–42]. During copulation, the *D. simulans* females also actively resist mounting attempts of *D. mauritiana* males. In the *D. mauritiana* × *D. simulans* cross, copulation duration is slightly shorter than that of *D. simulans* heterospecific crosses, but abnormally long compared to *D. mauritiana* conspecific crosses [40]. In the *D. simulans* × *D. sechellia* cross, copulation duration varies from as short as ~16 minutes to the normal ~25–30 minutes observed in *D. sechellia* conspecific crosses. In each heterospecific pair (including heterospecific crosses involving the other species in the group, *D. melanogaster*; [38]), the duration of copulation is more similar to the copulation duration typical of the male species. This suggests the possibility that differences in posterior lobe morphology might allow males to maintain copulation duration for times that are characteristic of that species, which could be important for successful insemination [38].

The second defect observed among these heterospecific crosses is abnormal sperm transfer and lower offspring production. In the *D. simulans* × *D. mauritiana* cross, a smaller fraction of the *D. mauritiana* sperm are stored by the *D. simulans* female compared to either pure species cross, and the cross produces 40% fewer offspring compared to pure species *D. mauritiana* and 70% fewer compared to pure species *D. simulans* [37, 40]. In the *D. mauritiana* × *D. simulans* cross, *D. simulans* males transfer an abnormally large number of sperm during copulation, however, the female loses the heterospecific sperm rapidly from her storage organs. Oviposition rates of mated females are reduced, and the cross yields fewer progeny [40]. Lastly, in the *D. simulans* × *D. sechellia* cross, *D. sechellia* males transfer few or no sperm to *D. simulans* females even when copulation duration last the full 30 minutes [40]. Although the morphology of the posterior lobe might contribute to these postinsemination reproductive defects, these reproductive fitness problems might also result from differences in molecular incompatibilities between male seminal fluid proteins and proteins in the female reproductive tract, which are known to cause postcopulatory reproductive problems between other *Drosophila* species [43, 44].

## 5. Genetics of Species Genital Morphology

Because of their rapidly evolving morphology and their importance in reproductive fitness, animal genitalia have attracted the attention of evolutionary geneticists. Aside from presenting a good model to study the genetics of rapidly evolving morphological traits, there is reason to suspect that genitalia might possess a particular genetic architecture if sexual selection drives the evolution of morphology within species [4]. In particular, we might expect that several genes would be necessary to specify differences in genital morphology (or in the case of sensory lock-and-key, differences in behavioral preferences) in both males and females, reflective of the step-wise coevolution of phenotypes between the sexes. We might also predict to find the molecular signature of selection at loci important for specifying morphological (behavioral) differences.

Many closely related species possess divergent genital structures thus making it possible to construct interspecific hybrid genotypes to dissect the genetics of genital morphology. Although experiments mapping differences in genital morphology have been performed in only two species groups where genitalia contribute to RI, the results of these mapping experiments suggest that the genetic architecture of species differences in genital morphology bears some similar characteristics across taxa. The results also support at least one of our predictions: species differences in genital morphology appear to be specified by many genes, and the phenotypic effects of species alleles act in the direction of the species phenotype, consistent with the idea that sexual selection drives the evolution of morphology within species. Moreover, the genomic locations that have large effects on morphological differences are similar among species within some species groups, which suggests the possibility that change at some of the same loci might be involved in the evolution of species-specific genital morphology.

Recent work on *C. maiyasanus* and *C. iwawakianus* has identified several genomic regions between these two species that carry loci specifying the species differences in both the male and female genital morphology involved in structural lock-and-key RI [45, 46]. Sasabe and colleagues measured a panel of reciprocal $F_1$ and backcross hybrids for genital morphology and performed quantitative trait loci (QTL) mapping experiments to identify the minimum number of genes that specify male and female morphology. They measured two genital phenotypes for each sex: in males, they measured copulatory piece length and copulatory piece width, and in females they measured vaginal appendix length and vaginal appendix width. The results of their mapping identified 15 QTL that reside across 8 of the 14 linkage groups in these species: three QTL specify differences in copulatory piece length, three QTL for copulatory piece width, four QTL for vaginal appendix length, and five QTL for vaginal appendix width.

Several genetic studies have also been performed that map the genomic regions that specify the differences in posterior lobe morphology among the *D. melanogaster* complex species. Because crosses to *D. melanogaster* usually result in dead or sterile offspring [47, 48], most of these mapping studies focus on comparisons among *D. mauritiana*, *D. sechellia*, and *D. simulans* where it is easier to obtain $F_2$ and backcross hybrid genotypes [49]. QTL mapping experiments between *D. mauritiana* and *D. simulans* identified 20 QTL that map across each of the major chromosomes in these species underlying the posterior lobe morphological differences [50]. In the *D. sechellia-D. simulans* species pair, QTL mapping revealed a minimum of 13 QTL that have effects on posterior lobe morphology [51]. An introgression mapping approach was used to map loci specifying morphology between *D. mauritiana* and *D. sechellia* to small genomic regions across roughly 50% of the genome. The mapping results identified a minimum of six regions with large effects

Table 1: Species hybridizations that display lock-and-key reproductive isolation.

| Order | Species hybridized | Lock-and-key mechanism | Reference(s) |
|---|---|---|---|
| Lepidoptera | *Malacosoma franconica* and *M. neustria* | Structural | Standfuss [15] |
| | *Deilephia porcellus* and *D. elpenor* | Structural | Federley [16] |
| | *Erebia nivalis* and *E. cassioides* | Sensory | Lorkovic [27] |
| Araneae | *Misumenops rothi* and *M. gabrielensis* | Structural | Schick [17] |
| | *Misumenops lepidus* and *M. californicus* | Structural | Schick [17] |
| Coleoptera | *Carabus iwawakianus* and *C. maiyasanus* | Structural | Sota and Kubota [18] |
| | *Macrodactylus costulatus*, *M. sylphis*, and *M. sericinus* | Sensory | Eberhard [3] |
| Polydesmida | *Parafontaria tonominea* sp. A and sp. B | Structural | Tanabe and Sota [21] |
| Zygoptera | *Sympecma*, *Lestes* (7 species total) | Sensory/structural | Loibl [31] |
| | *Ischnura elegans* with *Enallagma*, *Platycnemis*, *Sympecma*, *Lestes* (various species) | Sensory/structural | Krieger and Krieger-Loibl [32] |
| | *Argia*, *Enallagma*, *Ischnura*, *Telebasis* (10 species total) | Sensory/structural | Paulson [33] |
| Diptera | *Drosophila santomea* and *D. yakuba* | Structural | Kamimura and Mitsumoto [24] |
| | *Drosophila mauritiana*, *D. sechellia,* and *D. simulans* | Sensory | Robertson [42]<br>Cobb et al. [41]<br>Coyne [37]<br>Price et al. [40]<br>Jagadeeshan and Singh [38] |

on morphology [52]. Interestingly, some of these genetic regions have morphological effects on posterior lobe size, but not posterior lobe shape, whereas others have morphological effects on posterior lobe shape, but not posterior lobe size. This result suggests that these two posterior lobe phenotypes are specified, in part, by different loci. Transcriptome sequencing experiments in the larval tissue that gives rise to the male genitalia also reveal a possible role for gene expression differences in the insulin/insulin-like signaling pathway in specifying morphological differences between *D. mauritiana* and *D. sechellia* [52].

## 6. Conclusions and Prospects

Although it has long been thought that differences in genital morphology had little or no importance for speciation, it appears that in some hybridizations lock-and-key mechanisms do in fact contribute to RI (Table 1). However, despite the widespread diversity of genital morphologies among many animal taxa, it is clear that genitalia usually do not cause structural lock-and-key RI in the strict sense [4, 9]. In most species crosses where structural lock-and-key has been tested, it seems reasonable to suspect that the criteria for structural isolation were not satisfied—correlated differences of genital morphology between males and females within a species appear to occur much less frequently than the case of substantial diversity of male genital morphology among closely related species, but relatively little diversity of morphology among females of those species. This common sexual asymmetry in the degree of genital morphological divergence suggests the possibility that sensory lock-and-key RI mechanisms, however, could be quite common in limiting gene flow between species [4].

Thus, two major challenges face the study of genital evolution and its role in speciation for the near future. The first is to identify the frequency with which RI via genital sensory lock-and-key occurs among different taxa. This might be most easily tested in insect and arachnid species, although the potential for genital sensory lock-and-key RI in some vertebrate systems is currently being explored (B. Langerhans, personal communication, I. Schlupp, personal communication). The second challenge will be to dissect the mechanistic basis—both phenotypically and molecularly—of morphologically induced behaviors or physiological responses that result from genital incompatibilities. This problem will require the availability of sophisticated measurement and molecular tools to manipulate genital morphology, but some experimental systems such as *Carabus* and *Drosophila* appear poised to begin work on determining the sensory consequences of genital morphology and its effect on RI.

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*Review Article*

# Chromatin Evolution and Molecular Drive in Speciation

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Are there biological generalities that underlie hybrid sterility or inviability? Recently, around a dozen "speciation genes" have been identified mainly in *Drosophila*, and the biological functions of these genes are revealing molecular generalities. Major cases of hybrid sterility and inviability seem to result from chromatin evolution and molecular drive in speciation. Repetitive satellite DNAs within heterochromatin, especially at centromeres, evolve rapidly through molecular drive mechanisms (both meiotic and centromeric). Chromatin-binding proteins, therefore, must also evolve rapidly to maintain binding capability. As a result, chromatin binding proteins may not be able to interact with chromosomes from another species in a hybrid, causing hybrid sterility and inviability.

## 1. Introduction

Are there biological generalities that underlie hybrid sterility or inviability? In other words, do common mechanisms dictate that mules and leopons, for example, are sterile? The widely accepted Dobzhansky-Muller incompatibility (DMI) model of reproductive isolation [1, 2] does not provide an answer to this question. Instead, the DMI model only predicts that combinations of incompatible genes from different species lead to sterile or inviable hybrids. According to Mayr [3], reproductive isolation is an accidental byproduct of speciation. Recently, around a dozen "speciation genes" have been identified, and the biological functions of these genes are revealing molecular generalities that control hybrid sterility and inviability [4–8] (but see [9]). They are chromatin evolution and molecular drive in speciation.

Dover [10] argues, "In the case of many families of genes and noncoding sequences…, fixation of mutations within a population may proceed as a consequence of molecular mechanisms of turnover within the genome [i.e., molecular drive]. …There are circumstances in which the unusual concerted pattern of fixation permits the establishment of biological novelty and species discontinuities [i.e., reproductive isolation]…" Genes encoding heterochromatin proteins may have evolved rapidly to counteract mutations within repetitive DNA sequences in heterochromatin, which accumulate by molecular drive. The molecular drive theory once dominated the field of speciation, supported by the discovery that selfish transposable elements cause hybrid dysgenesis [11–14]. However, this hypothesis has been discounted, as there is no direct evidence that transposons are involved in reproductive isolation [15, 16] (but see [17, 18]). Even the most contemporary textbook concerning speciation [19] does not cite the Dover's [10].

## 2. *Lhr* and *Hmr* of *Drosophila*

When *Drosophila melanogaster* females mate with *Drosophila simulans* males, only weak, sterile, female hybrids eclose, as male hybrids die during larval stages [20]. Watanabe [21] discovered a *D. simulans* mutation, *Lethal hybrid rescue* (*Lhr*), that prevents hybrid larval lethality and restores female hybrid vigor [22]. It was thought that the wild-type allele of *D. simulans Lhr* was incompatible with X-linked genes from *D. melanogaster*. It has since been demonstrated that *Lhr* encodes a heterochromatin protein, HP3, which contains a boundary element-associated factor 32/Su(var)3-7/Stonewall (BESS) domain [23–25]. The X-linked *Hybrid male rescue* (*Hmr*) of *D. melanogaster* [26] has an effect similar to *Lhr* when mutated, and it also restores female hybrid fertility in this context [27]. *Hmr* encodes a DNA-binding protein with two myb/SANT-like in Adf-1 (MADF) domains [28].

LHR and HMR may physically interact through their BESS and MADF domains and may colocalize to specific chromatin regions. LHR also interacts with the heterochromatin proteins HP1 and HP6, as demonstrated by yeast two-hybrid (Y2H) experiments, RNA interference (RNAi) knockdown, and Bayesian network analysis [23, 25, 29–31]. The ratio of the number of nonsynonymous substitutions per nonsynonymous site to the number of synonymous substitutions per synonymous site ($K_a/K_s$) [32] and McDonald-Kreitman (MK) test [33] indicate that *Hmr* and a subset of genes encoding heterochromatin proteins (including *Lhr* and *HP6*) have evolved under positive selection [23, 28, 31, 34]. The involvement of *Lhr* and *Hmr* in reproductive isolation is reminiscent of speciation mediated by molecular drive. A comprehensive analysis of LHR, but not HMR, binding sites in the genome has been performed [35].

## 3. *zhr* of *Drosophila*

Involvement of heterochromatic repetitive sequences in hybrid inviability is evident when crosses between *D. simulans* females and *D. melanogaster* males (reciprocal to the cross discussed above) are analyzed. Progeny from this cross are sterile, male hybrids, as most female hybrids die during embryogenesis [20, 36]. We discovered *zygotic hybrid rescue* (*zhr*), a *D. melanogaster* gene that prevents female embryonic lethality in this context [37]. Genetic analyses using chromosome deficiencies and duplications [38–40] indicate that female hybrids are rescued if the number of 359-bp repetitive sequences (1.688 satellite) on the *D. melanogaster* X chromosome is decreased. In addition, hybrids of both sexes are inviable when repetitive sequences are added. In embryos from *D. simulans* mothers, chromatin regions rich in the 1.688 satellite are not properly condensed [41], resulting in mitotic defects such as chromosome bridges and irregularly spaced nuclei [41, 42].

The 1.688 satellite was one of the earliest sequences cloned in *Drosophila* [43, 44] and represents more than 4% of the *D. melanogaster* genome [45–47]. Related sequences are present in *D. simulans*, but the homology is low [48–51]. Heterochromatin regions rich in the 1.688 satellite may represent binding sites for the putative HMR/LHR complex. However, because *zhr* only affects hybrid viability when *D. simulans* females are crossed to *D. melanogaster* males (not the reciprocal cross), the larval and embryonic hybrid-inviability phenotypes associated with these crosses were thought to be independent (see [37, 52] for additional evidence). However, the possibility remains that female hybrids from *D. melanogaster* mothers are viable because proteins necessary to cope with *D. melanogaster* heterochromatin on the X chromosome are supplied maternally. This explanation is consistent with the model proposed by [53, 54]. Identification of proteins that bind to the 1.688 heterochromatin satellite will be informative [55–58]. *maternal hybrid rescue* (*mhr*) of *D. simulans* [52] and *Simulans hybrid females rescue* (*Shfr*) [59] represent loci encoding strong 1.688-binding candidates.

Although the 1.688 satellite does not seem to encode any proteins, it is transcribed in ovaries and silenced by the RNAi machinery. This silencing is mediated by repeat-associated small interfering RNA, also called Piwi-associated RNA [60]. In hybrids, failure to silence the 1.688 satellite may lead to heterochromatin decondensation and lethality [54]. Finally, the *hybrid lethal on the X* (*hlx*) locus of *D. mauritiana* affects viability of *D. simulans* hybrids and has been mapped to heterochromatin [61]. It will be interesting to determine whether this locus also consists of repetitive sequences, similar to *zhr*.

## 4. *OdsH* of *Drosophila*

In reciprocal crosses between *D. mauritiana* and *D. simulans*, female hybrids are fertile but male hybrids are sterile [62]. Many genes have been identified that affect this male hybrid sterility (for a review see [63]). These loci are scattered throughout the two genomes, but an X-linked gene, *Odysseus* (*Ods*), plays a particularly important role. When the *D. mauritiana* allele of *Ods* is cointrogressed with a closely linked gene onto the *D. simulans* genetic background, males become sterile [64, 65]. This hybrid male sterility gene has been isolated as *Ods-site homeobox* (*OdsH*) [66]. *OdsH* is paralogous to *uncoordinated-4* (*unc-4*), which is expressed in postmitotic neurons and epidermal cells [67]. In *Drosophila*, *OdsH* is thought to have arisen through gene duplication and neofunctionalization, thereby assuming a novel role in spermatogenesis [66, 68, 69]. Ample evidence suggests that *OdsH*, especially its DNA-binding homeodomain, has evolved under positive selection [66, 69]. Four genes downregulated in sterile male hybrids are thought to lie downstream of *OdsH* [70]. And misexpressed genes are disproportionately more common on autosomes than on the X in the males with *OdsH* introgression [71]. Regulatory regions of these genes may contain binding sites for the *OdsH* transcription factor.

Alternatively, but not mutually exclusively, Bayes and Malik [72] suggested that the ODSH protein localizes to evolutionarily dynamic loci in heterochromatin and that ODSH abundance and localization during premeiotic phases of spermatogenesis are different between *D. simulans* and *D. mauritiana*. ODSH from *D. mauritiana* associates with the heterochromatic Y chromosome of *D. simulans,* leading to decondensation and male hybrid sterility [72]. These data reveal that rapid heterochromatin evolution affects the onset of male hybrid sterility [72], in addition to hybrid inviability [37, 41]. However, it remains unclear which DNA sequences ODSH binds with the highest affinity.

## 5. *Nup160* and *Nup96* of *Drosophila*

The discovery of strains that restore the fertility of *D. simulans/D. melanogaster* female hybrids [73] provided the tools to introgress *D. simulans* chromosomal segments onto the *D. melanogaster* genetic background [74]. Both male and female introgression homozygotes successfully made were sterile, and the genes responsible for the male and female sterility have been mapped [75–77]. Among them, *Nucleoporin 160* (*Nup160*) of *D. simulans* was identified as

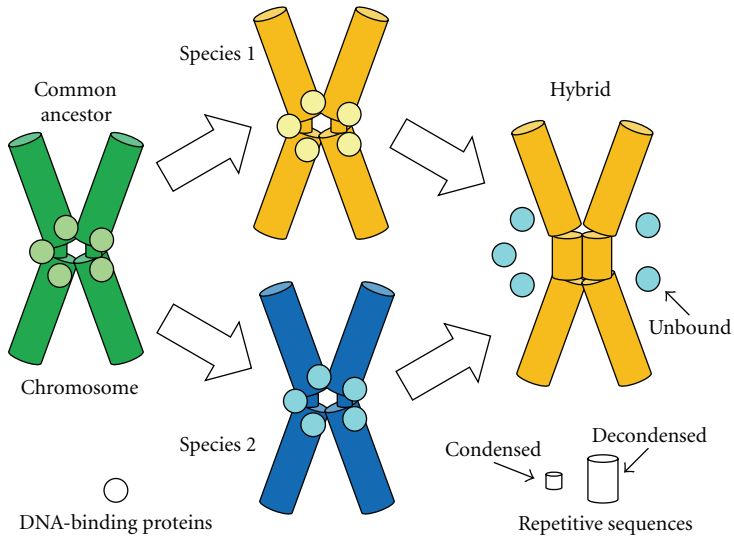


Figure 1: A hybrid sterility and inviability model based on chromatin evolution and molecular drive in speciation. Repetitive satellite DNAs evolve rapidly, thereby accelerating the evolution of chromatin-binding proteins (from the common ancestor to species 1 and species 2). Hybrids are sterile or inviable because the chromatin-binding proteins from species 2 cannot recognize the repetitive sequences of species 1.

the gene underlying female sterility on the *D. melanogaster* genetic background [78]. Both *D. simulans Nup160* and *Nucleoporin 96* (*Nup96*), which also encodes a component protein of the nuclear pore complex (NPC), cause inviability in *D. melanogaster/D. simulans* male hybrids [78–80]. This is independent of the $F_1$ hybrid inviability that can be rescued by *Lhr* mutation and is only revealed in introgression bearers or hemizygotes made from *D. melanogaster* deficiencies [81, 82].

Population genetics studies have indicated that positive selection is operating in seven nucleoporin genes, including *Nup160* and *Nup96* [79, 80, 83] and have revealed significant correlated evolution between them [84]. Several hypotheses have been proposed for why nucleoporins are evolving so rapidly in *Drosophila* [78–80, 83], but here I will focus on the hypothesis most highly related to the molecular drive theory. The NPC forms channels that allow transport of macromolecules between the nucleus and cytoplasm (for a recent review see [85]). In addition, NPC components also function in kinetochore/spindle formation and transcriptional regulation (i.e., dosage compensation) [86–91]. The evolution of scaffold nucleoporins (the NUP107-160 complex) may have accelerated to recognize repetitive sequences in centromeric heterochromatin. In this way, incompatible NPCs may result in hybrid sterility and inviability through improper kinetochore formation. Alternatively, small RNAs derived from repetitive DNA sequences may not be properly trafficked in cells with incompatible NPCs. This leads to chromatin decondensation and, ultimately, sterility or inviability. Such a model has been proposed in the meiotic drive system of *D. melanogaster* (see below). In this case, mislocalized and truncated Ran GTPase Activating Protein (RanGAP), which is encoded by *Segregation distortion* (*Sd*) [92], disrupts proper nuclear transport of small RNAs derived from *Responder* (*Rsp*) and ribonucleoprotein complexes that are required to suppress the *Rsp* satellites [54, 93].

## 6. *Prdm9* of Mice

Evidence for chromatin mechanisms in speciation is not restricted to *Drosophila*. In the cross between *Mus musculus musculus* and *M. m. domesticus*, female hybrids are fertile, but male hybrids are sterile (for a review see [94]; see also [95, 96]). Backcross analyses have indicated that three or more independently segregating loci are involved in this male hybrid sterility. One gene, *Hybrid sterility 1* (*Hst1*) of *M. m. domesticus*, is polymorphic: the $Hst1^s$ allele causes sterility, but $Hst1^f$ does not [97]. This situation is similar to the hybrid rescue mutations in *Drosophila*. The *Hst1* locus was mapped to the *PR domain zinc finger protein 9* (*Prdm9*) gene, where PR stands for PRDIBF1 and RIZ homology. *Prdm9* encodes a histone H3 lysine 4 (H3K4) trimethyltransferase [98], which is also known as the *Meisetz*, meiosis-induced factor containing a PR/SET domain and a zinc-finger motif [99]. Hybrid males sterilized by the *Prdm9* introgression exhibit frequent dissociation of the X and Y chromosomes during meiosis [98], similar to the sterile male hybrid from a cross between *M. m. musculus* and *M. spretus* [100–102]. A gene involved in *M. musculus*/*M. spretus* male hybrid sterility and a gene responsible for X-Y dissociation in *M. m. musculus*/*M. m. molossinus* hybrid males (the latter termed *Sex-chromosome association* (*Sxa*)) have been mapped to the pseudoautosomal region of the X chromosome [103, 104]. The heterochromatin content of this region is quantitatively different among species or subspecies [105, 106].

The DNA-binding domain of PRDM9 consists of multiple, tandem C2H2 zinc finger domains and is evolving rapidly under positive selection in diverse metazoans, including rodents and primates. Rapid evolution of this binding domain likely results from recurrent selection for binding specificity to satellite DNAs [107–109]. The interaction between PRDM9 and repetitive sequences also affects meiotic recombination [110–112]. Histone H3 modifications

Table 1: Hybrid incompatibility genes mentioned in the current paper. Whether data concerning these genes are consistent or inconsistent with the current hypothesis is indicated.

| Gene | Species | Phenotype[a] | Comment | Consistent | Reference |
|---|---|---|---|---|---|
| *Lhr* (HP3) | *Drosophila melanogaster/D. simulans* | $F_1$-L | Interaction with heterochromatin proteins | Yes | [23, 24] |
| *Hmr* | *D. melanogaster/D. simulans* | $F_1$-L, FS | Chromatin-binding | Yes | [28] |
| *zhr* (1.688) | *D. melanogaster/D. simulans* | $F_1$-L | Centromeric repetitive DNA | Yes | [37, 41] |
| *hlx* | *D. melanogaster/D. simulans* | BC-L | Centromeric repetitive DNA? | Yes | [61] |
| *OdsH* | *D. melanogaster/D. simulans* | $F_1$, BC-MS | Heterochromatin-binding | Yes | [66, 72] |
| *Nup160* | *D. melanogaster/D. simulans* | BC-L, FS | Centromeric heterochromatin-binding? | Yes | [78, 80] |
| *Nup96* | *D. melanogaster/D. simulans* | BC-L | Centromeric heterochromatin-binding? | Yes | [79] |
| *Prdm9* | *Mus m. musculus/M. m. domesticus* | $F_1$, BC-MS | Histone methylation | Yes | [98] |
| *Sxa* | *M. m. musculus/M. m. domesticus; M. musculus/M. spretus* | $F_1$, BC-XY, MS | Heterochromatic repetitive DNA? | Yes | [103, 104] |
| *tmy* | *D. simulans/D. mauritiana* | BC-MS | Not separable from the gene causing meiotic drive | Yes | [120] |
| *Ovd* | *D. p. pseudoobscura/D. p. bogotana* | $F_1$, BC-MS | Chromatin-binding; also causing meiotic drive | Yes | [122] |
| Cent728 | *Mimulus guttatus/M. nasutus* | $F_1$, BC-FMD | Centromeric repetitive DNA | Yes | [130] |
| *JYalpha* | *D. melanogaster/D. simulans* | BC-MS | Transposition | No | [133] |

[a] $F_1$: hybrid; BC: (equivalent to) backcross; L: lethal; FS: female sterile; MS: male sterile; XY: XY dissociation; FMD: female meiotic drive.

are typical epigenetic events that determine chromatin status (for reviews see [113, 114]). Genomic regions characterized by heterochromatin-mediated gene silencing are rich in histone H3K9 methylation and have few histone acetylations. In contrast, histones in transcriptionally active euchromatic regions are highly acetylated and methylated at H3K4. Interestingly, chromatin structures regulated by H3K9 methylation, Su(var)3-9, HP1, or the RNAi pathway are required to maintain the structural integrity of tandemly repeated, heterochromatic sequences, like the 1.688 satellite, in *D. melanogaster* [115].

## 7. Three Drives in Speciation

The meiotic drive model of male hybrid sterility assumes an arms race between meiotic drive genes and suppressor genes in which male hybrids exhibit segregation distortion or sterility if they inherit drive genes, but not their corresponding suppressors [116, 117]. At first, this model was not accepted because cryptic segregation distortion was not detected in interspecies crosses of *Drosophila* [118, 119]. In the cross between *D. mauritiana* and *D. simulans*, one gene involved in male hybrid sterility is not separable from the meiotic drive gene, *too much yin* (*tmy*), by recombination [120]. In addition, the gene *Overdrive* (*Ovd*) causes both male hybrid sterility and meiotic drive in aged males when *D. pseudoobscura pseudoobscura* is crossed with *D. p. bogotana* [121, 122]. Interestingly, *Ovd* encodes a protein that contains a MADF DNA-binding domain [122], similar to HMR of *D. melanogaster* [28].

In the context of speciation, meiotic drive can be the manifestation of molecular drive. The most common example of this phenomenon is centromere drive. The centromere drive model assumes that both DNA and protein components of centromeric chromatin are evolving rapidly and that incompatibilities between rapidly evolving centromeric components may be responsible for hybrid sterility [123]. In particular, the expansion of centromeric repetitive sequences provides more microtubule attachment sites, thereby creating a stronger centromere that tends to be included in the oocyte nucleus [123]. This represents an alternative force from molecular drive that is distinct from a variety of mutational processes that include replication slippage, unequal exchange, transposition, and excision [10, 124–126]. To suppress potential nondisjunction of chromosomes that carry expanded satellite DNAs, the gene *centromere identifier* (*cid*) has evolved rapidly in diverse organisms including *Drosophila* [127, 128]. *cid* encodes centromeric histone H3-like, a homologue of human Centromere protein A (CENP-A). Examples of centromeric repeats affecting meiotic drive include the *Rsp* locus of *D. melanogaster*, which is the target of *Sd* [129], and the Cent728 repeat, which is responsible for female meiotic drive in the Monkeyflower hybrid between *Mimulus guttatus* and *Mimulus nasutus* [130].

## 8. Applicability and Related Issues

Above I proposed a theory that hybrid sterility and inviability are generally the manifestation of chromatin evolution and molecular drive in the context of speciation, but I do not claim that this model explains every case. Among hybrid incompatibility genes discussed in recent review papers, only 10 of 18 (Table 1 of [5]), 8 of 14 (Table 1 of [6]), and 7 of 14 (Table S1 of [9]) are consistent with this theory. In addition, as most hybrid incompatibility data are from *Drosophila*, a different trend may appear if reproductive isolation genes are

identified from diverse taxa. A famous exception to this theory involves the *JYalpha* gene in *Drosophila*. *JYalpha* encodes a protein with sodium/potassium-exchanging ATPase activity and is located on chromosome 4 in *D. melanogaster* but on chromosome 3 in *D. simulans*. Therefore, males carrying homozygous introgression of *D. simulans* chromosome 4 on the *D. melanogaster* genetic background are sterile, as they do not inherit *JYalpha* from either species [131–133]. This is an example of male hybrid sterility caused by gene transposition between species, which is consistent with the gene duplication and nonfunctionalization model of speciation [134].

Haldane's rule is generally observed when hybrid sterility and inviability are encountered. This rule states that "when in the $F_1$ offspring of two different animal races one sex is absent, rare, or sterile, that sex is the heterozygous [heterogametic (XY or ZW)] sex" [135]. This rule is empirical and seems to be a composite phenomenon [136–138], although the dominance theory is applicable in most cases [139]. Here I propose an additional explanation for Haldane's rule, based on chromatin evolution and molecular drive in speciation. In hybrid animals, chromatin-binding proteins supplied from one species may not be able to recognize the other species' Y or W chromosome, as these chromosomes are generally heterochromatic and have high levels of repetitive satellite DNAs. This results in meiotic or mitotic chromosome decondensation or nondisjunction and leads to hybrid sterility or inviability in the heterogametic sex.

There are several chromatin state systems that have not been discussed yet, which may be related to the present issue. First, inactivation of the X chromosome in primary spermatocytes is necessary for the normal progression of spermatogenesis in heterogametic (XY) males [140] (but see [141, 142]), a process termed meiotic sex chromosome inactivation (MSCI). In some cases, male hybrid sterility may result from ineffective MSCI, as DNA-binding proteins may not be able to recognize and inactivate X chromosomes from different species (e.g., [63, 102]). Second, genomic imprinting affects a subset of genes, resulting in monoallelic and parent-of-origin-specific expression. This process usually depends on DNA methylation or histone modification (e.g., [143–146]). Species-specific variations in epigenetic marks may disrupt imprinting and lead to hybrid inviability. This can explain classic observations of unilateral incompatibility in rodent and flowering plant species (e.g., [147–150]).

## 9. Conclusion

As has been discussed in this paper, major cases of hybrid sterility and inviability seem to result from chromatin evolution and molecular drive in speciation (Table 1). Repetitive satellite DNAs within heterochromatin, especially at centromeres, evolve rapidly through molecular drive mechanisms (both meiotic and centromeric). Chromatin-binding proteins, therefore, must also evolve rapidly to maintain binding capability. As a result, chromatin-binding proteins may not be able to interact with chromosomes from another species in a hybrid, causing hybrid sterility and inviability (Figure 1).

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